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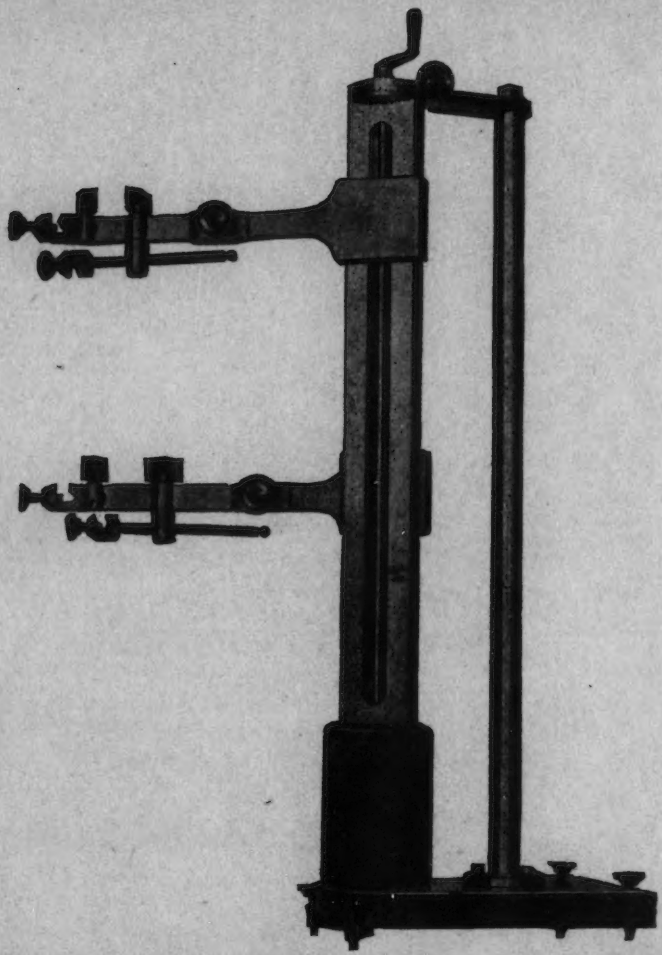

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STUDIES IN THE METABOLISM OF ALUMINIUM

I. Method for Determination of Small Amounts of Aluminium in Biological Material[1]

FRANK P. UNDERHILL AND F. I. PETERMAN

From the Department of Pharmacology and Toxicology, Yale University, New Haven, Conn.

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The chief interest in the metabolism of aluminium arises from its use as a constituent of food. The controversy between those who believe that aluminium compounds added to foods are harmful and those who take the opposite view has never been settled. The present investigation, begun in 1922, was undertaken with the conviction that the methods then published for the estimation of aluminium were so crude and inaccurate that the conclusions drawn are open to serious criticism. Accordingly a delicate and accurate method has been devised and by its use new facts on this problem have been established.

Two books have recently appeared which review the present status of the aluminium problem. E. E. Smith (1), devoting himself especially to aluminium as a native or added constituent of foods, summarizes investigations bearing on this subject. Stoklasa (2), after forty years of work on the distribution of aluminium in nature, publishes his results. No attempt is made here to review in detail this mass of material. The reader is referred to these two books for a more comprehensive survey.

The present status of the problem may be summarized as follows: Aluminium occurs widely distributed in plants. In general hydrophytes contain more than mesophytes or xerophytes. The roots act as reservoirs for the aluminium which is required in the economy of the plant. Seeds and blossoms also contain aluminium. In the former it is supposed to be in some way related to germination; in the latter to color.

[1] Constructed from a dissertation presented to Yale University in June 1925, by F. I. Peterman in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The feathers of birds contain considerable quantities of aluminium. Various investigators have reported its absence from or presence in the tissues of man and animals, wherein it is considered variously a noxious substance, a harmless contamination, or a vital biologic catalyser.

There is no doubt that significant quantities of aluminium may find their way into the body in natural foods and potable waters. Here they may be, in large measure, soluble in the gastric juice. Many widely used baking powders contain aluminium. Metallic aluminium, used as a container for foods, can scarcely be considered a source from which any significant amount can find its way into the body (3) (4).

Soluble aluminium compounds in low concentrations generally stimulate the growth of plants; sometimes to a remarkable extent (Stoklasa). High concentrations are decidedly toxic (5). The toxicity of certain soils is attributed to soluble iron and aluminium (5) (6) (7) (8).

Aluminium solutions are toxic to fish and frogs (partial immersion) a solution of more than 0.5 per cent of aluminium sulfate killing fish in a few hours (9) (10) (11). This effect has been attributed to the acidity of the solution (9) (10). A double citrate of aluminium injected into the lymph sac of the frog (12) results in general paralysis due to action on the ganglia of the brain and spinal cord. Longitudinal conduction is unimpaired. Metallic aluminium administered to pigeons is moderately toxic (13).

In higher animals and man, small amounts of aluminium compounds, introduced per os are without noticeable effect. Very large amounts have, in a few cases, produced severe poisoning and even death.

The principal interest in aluminium, recently, is its rôle as a constituent of foods and its possible deleterious effect upon the health of man. Smith reviews the evidence on this point and concludes that aluminium compounds added to foods have never been shown in any definite case to have resulted harmfully. The evidence for absorption of aluminium is still indecisive. On the one hand, Myers (14) and his collaborators claim to have demonstrated the absorption of aluminium, and on the other a recent investigation, by McCollum, Rask and Becker (15) by means of the spectrograph draws the conclusions that "aluminium is not a constituent of either plant or animal matter," and is "not absorbed from the intestinal tract when present in the diet." Even more recently Wright and Papish (16) also employing the spectrographic method have shown the presence of aluminium in cow's milk, and Kahlenberg and Closs (16a) demonstrated its existence in a variety of food products.

THE ANALYTICAL METHOD. The method employed for determining the aluminium content of blood and tissues originated in a suggestion of Atack (17). Alizarine, in the presence of aluminium, forms a red compound, the well known alizarine lake, which is a very sensitive tes

aluminium. By carefully controlling the conditions under which this compound is formed, it may be used for the quantitative estimation of small amounts of this metal. Atack's method, as outlined, has been thoroughly investigated and found to be unsatisfactory, for reasons which will be detailed later. It has also been reported upon unfavorably by Hatfield (18) and Wolff (19).

Since the beginning of the investigation the method has undergone modifications from time to time as experience indicated. As finally evolved, and as used at present, it is as follows: The ash of the blood or tissue is dissolved in 5 cc. of 10 per cent sulfuric acid and the volume made to 50 cc. with distilled water. In order to remove iron which interferes two 5 cc. portions of this solution are pipetted into 30 cc. separatory funnels of cylindrical shape, 0.1 to 1 cc. of 60 per cent ammonium thiocyanate is added and the ferric thiocyanate removed by "shaking out" with successive portions of ether, 10 cc. being used for the first two shakings, and 5 cc. for each of those following. The ether layer, in which the iron compound dissolves easily, is removed by suction.

Then 5 cc. portions of the clear, colorless solution are transferred to 25 cc. volumetric flasks. To each is added 2.5 cc. of a mixture of 4 parts of glycerine to 1 part of 10 per cent citric acid, followed by 1 drop of 0.5 per cent mono sodium alizarine sulphonate ("Alizarine Red S"). Appropriate standards are prepared at the same time and are treated in exactly the same manner. The solutions are now neutralized by bubbling ammonia vapor through them, the alizarine acting as indicator, until the *first* more-pink-than-yellow color appears. All the flasks, having an equal volume of solution, are carefully adjusted to the same color. Then to each is added 0.5 cc. of the 0.5 per cent sodium alizarine sulphonate, the solutions mixed, and 0.2 cc. of concentrated ammonium hydroxide added. The necks of the flasks are washed with a little distilled water and the solutions are mixed again. The flasks are stoppered and allowed to stand 24 hours, when 0.5 cc. of glacial acetic acid is added and the volume made to the mark with distilled water. The solutions are thoroughly mixed by inversion and shaking and the colors compared in a colorimeter against the proper standard or standards.

DETAILED DISCUSSION OF THE METHOD. *Preparation of samples. A. Blood.* Ten to 20 cc. of blood were used to prepare the ash. This is more than is necessary for a single determination of aluminium, but it was considered desirable to have such a quantity that analyses could be repeated if necessary. For a single estimation 2 cc. of blood are sufficient. The analyses in this work were made on the equivalent of 1 to 2 cc. of blood, save when humans were the experimental subjects, in which case less blood was used.

The fresh blood, usually oxalated, is measured into a 40 cc. silica crucible

and dried in an oven at about 110°C. for 24 hours. It is then placed in an electric muffle furnace at the lowest temperature, with the door partly open. The initial charring must take place slowly, otherwise the material foams and swells enormously, overflows the crucible and part is inevitably lost. After the initial slow charring is finished, and there is no more danger of overflowing, the muffle is closed to complete the ashing. By this means a fluffy yellow or reddish brown residue is obtained which dissolves easily in the small amount of acid used. If a high temperature is employed the ash fuses, seemingly into the crucible, and dissolves with difficulty, and never completely.

When the ashing is finished, (about 24 hours) the crucible is cooled, 4 cc. of 10 per cent sulfuric acid are added dropwise, so that the entire contents are moistened and washed to the bottom of the crucible. A watch glass is used to cover the mixture while it is boiled until clear on a hot plate. The solution, having a very small volume at this point, is diluted and transferred to a 50 cc. volumetric flask. The crucible is rinsed with distilled water, then with a hot solution containing 1 cc. of 10 per cent sulfuric acid and finally thoroughly washed with distilled water. The contents of the flask are cooled and made to volume. The solution is mixed and immediately transferred to a paraffined bottle. Five cubic centimeters of this solution are used for the analysis as outlined above.

It was hoped that a blood filtrate, such as is ordinarily used for the determination of constituents of blood, could be employed. Trials showed that the sodium tungstate-sulfuric acid precipitant gives a red coloration with the alizarine. The trichloracetic acid filtrate always contains iron, in such a form that it is very difficult to extract with ether. The blood mixture must be centrifuged as filtering may dissolve appreciable quantities of aluminium from the filter paper. It was found that quantities of aluminium in the filtrates differed considerably but not constantly, from the amounts found in the ash. This method was finally given up as introducing more unknown factors than the direct ashing of the blood.

B. Tissues. The tissues were removed carefully (after the animal had been exsanguinated) weighed, washed in distilled water, placed in clean glass or porcelain containers, and dried at 110°C. for 24 to 48 hours. After thorough drying they were weighed again, and a small aliquot taken for analysis. In the early work, the whole organ was dried; in later work only the portion considered necessary was used. The weight of dry tissue used for analysis is about 1 gram. Various weights were used, but this one was finally adopted. The dry aliquot represents roughly 3 to 5 grams of wet tissue. The weight of dried bile varied from 0.1 to 1 gram.

The ease with which the dried white fluffy ash is obtained differs with the tissue used. Bile and lung usually ash quickly, as do some of the other tissues. Liver ashes slowly and sometimes with difficulty. However,

usually a white, light ash is obtained in 24 to 48 hours, at the lowest furnace heat.

It is important that the ashing take place at a low temperature. Tissues can be quickly reduced to a greenish molten material free of carbon if a high temperature is used. The ash is soluble with difficulty. The process proves ruinous to silica crucibles which "chip off" during the acid extraction and are soon rendered useless. The temperature used is even below that spoken of as a "low red heat." It is also possible that at a high temperature some aluminium might be lost.

Silica crucibles, known as "Vitreosil," were used for the ashing and proved very satisfactory. Porcelain crucibles, which were tried in some cases, gave impossibly high results, showing that comparatively large amounts of aluminium may be extracted from the porcelain. Blank determinations to find whether the silica crucibles would yield traces of aluminium in the process of ashing and extracting gave negligible results. They do, however, "chip" after they have been heated a number of times. They are easily cleaned with a mixture of hydrochloric and nitric acids. This was done each time the crucibles were used.

A silica core was used in the furnace to prevent contamination by fire clay with which the oven is lined.

"Wet ashing"—that is, digestion with sulfuric and nitric acids—was attempted a few times. Appreciable quantities of aluminium are dissolved from glassware during an acid digestion. Wolff (19) has also observed this. If decomposition by means of acid is desired, silica flasks should be employed. The acids should be redistilled from silica retorts because most samples of nitric and sulfuric acids contain, in the amounts used, significant quantities of aluminium (Wolff). Finally all nitric acid must be removed, as even in small quantities it causes the destruction of the final coloration (Atack).

Every precaution, in each step of the process, was taken to protect samples from contamination.

CRITIQUE OF THE METHOD. In this colorimetric method for the determination of aluminium there are three important departures from that suggested by Atack. These are 1, the removal of iron; 2, the regulation of the hydrogen ion concentration; 3, the time element. Other minor differences occur, but these are the most significant.

Removal of iron. Because iron and aluminium are very closely related chemically and are similar in many of their general reactions, one would expect that iron, occurring in the same solution with, and in greater quantities than, aluminium would interefere with the quantitative estimation of the latter. As a matter of fact, this is the chief source of difficulty in all the aluminium methods. Atack states that "iron and chromium may be prevented from interfering by conversion into soluble citrates by

the addition of a citrate to the acidified solution before adding ammonia; in this manner it was found that the color given by 0.015 mgm. of aluminium was not affected by the presence of 10 mgm. of iron."

On the contrary, we have found that iron, even in small amounts, interferes markedly with the color produced with aluminium and alizarine S. iron, in the presence of the dye, gives a purple color, or precipitate, depending on the quantity, upon addition of ammonia. When the solution is finally acidified, a dirty brown color remains which completely masks the color of the aluminium compound, and renders it impossible to match against a pure standard. Conversion of iron to various types of complex and double salts was attempted but without success. Citric acid will prevent the interference of small traces of iron, and is used for this purpose as a matter of routine, but for any appreciable quantities it is useless. Citrates are not so useful as citric acid.

It became apparent that for any clear-cut determination of aluminium in biological material the iron must be eliminated. This was done by converting it to ferric thiocyanate and shaking out with ether.

This reaction is exceedingly interesting and useful. There are many conditions which influence its application. Ferric thiocyanate is reduced to ferrous thiocyanate with an appreciable velocity at ordinary temperature. The thiocyanate itself is oxidized, the products being H_2SO_4, CO_2, NH_3 and traces of HCN. This reaction is more rapid when the thiocyanate is in excess, as it is here, than when the ferric salt is in excess (20). Solutions which stood for several weeks showed distinct traces of H_2S. Furthermore, ferric thiocyanate in the presence of an excess of thiocyanate forms a complex ferrithiocyanate which is insoluble in ether (21). Consequently if this reaction is to be used successfully the iron salt must be removed at once.

The solubility of the ferric thiocyanate in the water and ether phases is also affected by several factors: by the amount of thiocyanate in excess, by the acidity, by the amount of ether used and especially by the quality of the ether itself. Any ether will remove the greater bulk of the iron but the final traces are difficult to extract. Anhydrous ether will not remove these final traces. The solution will be clear and colorless but upon standing a few hours will develop a pink color showing the presence of iron. Or a solution from which presumably all the iron had been extracted, if shaken out again with a different ether will show a considerable amount of iron.

Traces of substances present in the ether are also active in affecting the equilibrium between the two phases. Ordinary ether for anesthesia, containing from 1 to 4 per cent of alcohol is better for extraction than anhydrous ether. Old ether is also good. Ether which has been exposed to sunlight for a day or so is still better. With this, all the iron is removed

quickly with four, rarely five, extractions. Ether, after exposure to sunlight, contains peroxides (22, 23). Apparently these traces of foreign substances control the equilibrium between the water and ether phases to a marked extent. Finally, as a routine procedure, ether for anesthesia, or anhydrous ether to which alcohol has been added, is left in the sunlight for a day or two before using. Occasionally it is necessary to redistill ether before using, in which case it is redistilled from lime, and treated as anhydrous ether.

Iron is more easily removed if the first two shakings are made with larger volumes of ether than solution. These first extractions remove by far the greater quantity of iron. After that, smaller amounts of ether are sufficient.

Ammonium thiocyanate must be present in fairly large excess; otherwise the iron is difficult to extract. The amount used depends on the amount of iron present in the material. Solutions of bile, lymph and urine may usually be analysed without shaking out with thiocyanate. This shortens the process considerably, 0.2 cc. of thiocyanate is generally enough for the iron in lung and 0.7 to 0.8 cc. for blood and liver. Solutions must be slightly acid.

The thiocyanate must be prepared fresh at intervals of a week or so. When old, or after continued exposure to light, the excess of this reagent remaining in the solutions for analysis will decompose with the precipitation of sulphur. This precipitate interferes with the passage of light through the final solution, causing the colorimeter reading to indicate more aluminium than is present by making the solution appear darker. Thiocyanate is therefore kept in the dark when not in use.

Adjustment of hydrogen ion concentration. In presenting his method for the estimation of aluminium, Atack apparently took no account of the fact that sodium alizarine sulphonate is an acid-base indicator, and that its color depends upon the hydrogen ion concentration of the solution. Salm (24) gives the range for this dye as yellowish green ($H^+ = 10^{-2}$), brown ($H^+ = 10^{-5}$), red ($H^+ = 10^{-6}$), lilac ($H^+ = 10^{-11}$), violet ($H^+ = 10^{-12}$). We have always designated the color in acid solutions as "lemon yellow" and have never observed the brown mentioned by Salm when solutions were iron-free. At $H^+ = 10^{-5}$ the color is yellowish red. The aluminium alizarine sulphonate is red and difficult or impossible to distinguish from the color of the indicator at its red point. It is obvious that unless carefully controlled, the change in color, which is perhaps due only to change in H^+ may be considered due to the metal. The standard and unknown must be at the same H^+ concentration when comparison is made, if the color difference is to mean anything.

Atack evidently lost sight of these considerations. He used 5 cc. of a 1 per cent alizarine S; "the solution (is) made up to about 40 cc. with water

and then rendered slightly ammoniacal. After standing for five minutes it is acidified with dilute acetic acid, the alizarine S acting as indicator, acetic acid being added until no further change in coloration occurs." As a matter of fact, continued addition of acid causes a progressive change in color. One has no means of knowing when the addition of acid should cease because there is no sharp color change, merely a gradual transition from purple into red, to red mixed with yellow to lemon yellow. Then, also, the excess of acid gradually attacks the aluminium alizarine compound. Atack's failure to give specific limits to acidity and basicity leaves room for great variation in color which would be interpreted as difference in aluminium content.

By means of a series of buffer solutions prepared according to the method of McIlvaine (25) it was found that the point at which the red color disappears from the indicator is pH = 3.6 to 3.8, slightly less acid solutions showing a pink tinge. When, therefore, solutions are finally compared, the pH must be 3.6 to 3.8 or below. In order for all solutions to be at the same pH at the end, there must be an adjustment at some point, and thereafter the pH in every solution must be the same. Otherwise the results are not comparable.

In the method developed for this research, the regulation is made, as outlined, after all reagents except ammonia and the final acetic acid have been added. One drop of indicator is used and all solutions adjusted to the same color. This is much easier before than after the addition of the larger quantity of dye. The pH at this point is about 5.0. Thereafter, changes in pH are exactly the same in all solutions. Ammonium citrate, formed during neutralization has a slight buffering effect and cares for traces of iron. The small quantity of aluminium present does not influence the pH.

Atack used a relatively large quantity of alizarine S (5 cc. of a 1 per cent solution = 50 mgm. in 50 cc.). He states that a very large excess is essential, and that the color in acid solutions is not of sufficient intensity to interfere. It should, however, be noted that the final colors, compared in the colorimeter at pH = 3.6, are not pure colors. In any of these colored solutions there are always two components: the yellow of the excess indicator and the red of the aluminium alizarine compound. It is the predominance of the second over the first which is the determining factor. A large excess of the dye tends to obscure the latter component.

If the aluminium alizarine complex formed is actually the normal aluminium salt of alizarine sulphonic acid, and not a lake (Atack), the compound contains 1 part of aluminium to 35.15 parts of alizarine sulphonate. In the earlier parts of this research the standards ranged between 0.003 and 0.015 mgm. of aluminium, which should combine with 0.11 to 0.53 mgm. of alizarine sulphonate. At present the more usual range is

0.0005 to 0.005 mgm. of aluminium combining with 0.017 to 0.17 mgm. of alizarine. Granting that a large excess must be present, 50 mgm. seems an inordinately large amount. The quantity used in this research, originally 5 mgm., later 2.5 mgm. is many times that required by the constitution of the compound and has proved much better than the larger amount.

It has been found that colors do not develop sufficiently, in the five minutes recommended by Atack, to be distinguishable after acidification. The colors are very pale. Upon standing, they gradually deepen, so that after one hour they are distinguishable but become darker if allowed to stand longer. Three hours were found to give the maximum differences in color between standards of varying aluminium content. After that, colors continue to deepen but the ratio does not change. At present, the routine practice is to allow them to stand 24 hours. They are always read by daylight (northern exposure).

In fairness it should be pointed out that specimens of alizarine S purchased on the market vary greatly. It is possible that the specimen used by Atack was sufficiently different from those obtainable on the American market to account for some of the differences in results reported. Many different samples have been tested in this research and marked variation has been noticed, especially in regard to color, sensitivity to changes in pH, and color with aluminium. The one finally chosen for the research as being most satisfactory for the purpose is that supplied by Coleman & Bell Company.

Standards. The standard used is aluminium sulphate crystallized with 18 molecules of water, "Bakers' Analysed" having total impurities of 0.009 per cent (label). It is unpractical to attempt to purify this further, as the error in the standard used is infinitesimal; 1.23 grams of this salt are dissolved in 1 liter of water. This stock solution contains 0.1 mgm. of aluminium per 1 cc. From this is prepared the standard used which contains 0.001 mgm. per 1 cc. Standards used contain 0.0005, 0.001, 0.002, 0.003 and 0.005 mgm. of aluminium. Occasionally larger amounts are necessary, when a solution containing 0.01 mgm. of aluminium per 1 cc. is employed.

Four or five standards are always prepared as well as a blank containing only distilled water. To these is added H_2SO_4 equivalent to that contained in the unknowns. The reason for this will be discussed later.

It is necessary to prepare more than one standard. The colors formed are not exactly proportional to the concentration of the aluminium alizarine compound. If this compound were present alone this should be the case, in dilute solutions. But there is also the excess of alizarine which affects the color proportionality.

The chart shows the deviation from the theoretical color curve for a pure color. If the concentration of standard and unknown are close together

the deviation is very slight, but as the difference in concentration increases the deviation becomes greater. Consequently to make the results as accurate as possible standards having small differences in concentration are desirable. The unknown is compared against the standard nearest it in color. It often happens that the unknown concentration lies midway between two standards, in which case the average of the two results is taken.

The blank, prepared always as a check on the purity of the reagents and distilled water, is markedly different from the lowest standard and shows a difference of 2 to 3 millimeters when read in the colorimeter.

Standards are kept in paraffined bottles, as are all solutions used in connection with this method, excepting only the acetic acid.

Sensitivity. As used at present the range of this method is best between 0.0005 and 0.005 mgm. of aluminium. With suitable modifications it can be used to determine amounts as low as 0.00025 mgm. Here extreme care must be exercised at every step as colors are very delicate. They may, however, be read in a colorimeter. Probably even smaller amounts could be determined by comparing the colors in standard Nessler tubes. If more alizarine is used the colorimeter range may be extended up to 0.02 mgm. However, these colors are very dark, and tend to decrease in color difference with equal added increments of aluminium. Furthermore the aluminium alizarine complex tends to precipitate.

Temperature and light. Temperature and exposure to light seem to have little or no effect on the colors produced. However, after they have been acidified with acetic acid they must be compared at once as the acid gradually attacks the colored substance (Atack). The standards fade slightly more rapidly than the unknowns, so that it is inadvisable to run more than 3 or 4 unknowns, with duplicates, in a series.

Other details. Blank determinations, using distilled water instead of blood, evaporating, ashing, dissolving and treating with the same reagents showed that aluminium from this source is extremely small.

In each determination, besides the blank which is considered as one of the standards, is another, in duplicate, which is treated identically as the ash solutions are treated. That is, it contains an equivalent amount of acid, is treated with NH_4CNS and ether simultaneously with the other unknowns of that "set." If this blank shows an appreciable amount of aluminium the amount is subtracted from that of the unknowns or the solutions are considered contaminated and the determination repeated.

Originally HCl was used to dissolve the ashes. This was discarded because of the volatility of both the acid and the $AlCl_3$ formed and the probability of losing some of the latter during the boiling of the ash solutions. At present Merck's Concentrated H_2SO_4 is used. Because it contains traces of aluminium, and it is impracticable to try to purify it

further, an amount equivalent to that in the ash solution under examination is always added to the standards.

The addition of glycerine serves to keep the inorganic ions in solution when ammonia is added. It also prevents precipitation of the aluminium compound. Whether glycerine plays a part in the formation of the final color is not decided. Determinations on pure standards without glycerine give fainter and more variable colors. Glycerine is, therefore, always used.

The water used in every solution is redistilled from old Pyrex apparatus.

All determinations are done in duplicate.

Flasks must be identical in size, shape and color of glass.

Inorganic ions. The quantities of inorganic ions occurring in the amounts of blood and tissues used do not interfere with this determination. In relatively large quantities, calcium and magnesium cause precipitation of a blue alizarine salt in alkaline solutions which dissolves when the solution is acidified, and becomes colorless. Phosphates in large quantities interfere. Manganese gives a red color with alizarine. Boron as well as manganese is present in such small traces, if at all, that it has no influence on the color.

Other applications. This method is applicable to substances other than blood and tissues. Among other things it can be applied to foods, water, urine and feces. In this laboratory it has been applied to the determination of aluminium in fruits, vegetables, milk, bread, gastric juice, urine, and lymph. The results obtained will be embodied in subsequent publications. In certain cases minor modifications are necessary in order to adapt the method to the particular characteristics of the substance analysed. These specific details will be discussed at the proper time.

The table illustrates the relation of the color to the concentration. The figures are based on averages of many determinations on pure standards.

The results are plotted on the accompanying graph in which colorimetric reading is plotted against concentration. The smooth curve is the theoretical color relation for a pure color. As the concentrations differ

TABLE 1

The relation of aluminium concentrations to colorimetric readings

STANDARD AT 20 MM.	0.001	0.002	0.003	0.005	0.006	0.007	0.008	0.009	0.01	0.011	0.012	0.014
0.005	26.3		23.3	20.1		17.9			15.8			
0.003	23.9	22.6	20.1	18.0		16.2						
0.01					26.0	23.4	22.2	21.9	20.0	19.1	18.5	18.3

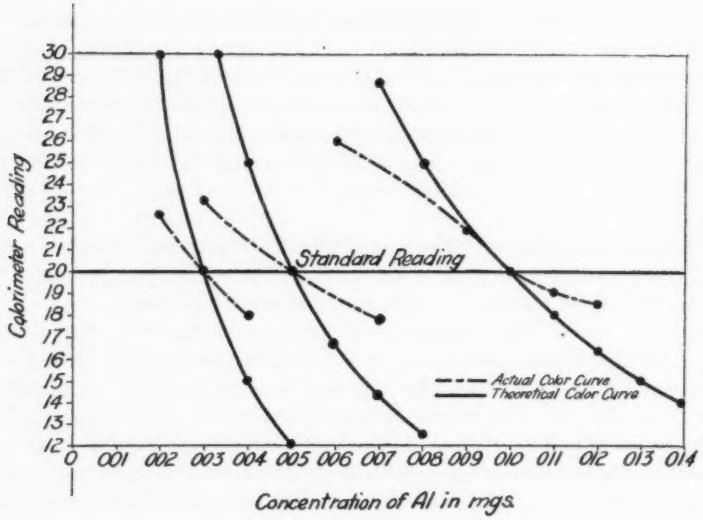

Fig. 1. Curve showing the relation between the theoretical color curve and the aluminium-alizarine color.

more from that of the standard the experimental curve diverges. A set of such curves may be drawn corresponding with each standard used for comparison. Three are given for solutions containing 0.003, 0.005 and 0.01 mgm. of aluminium. It is seen that if the colorimetric readings do not differ greatly from the reading of the standard the error is small, but when wide differences occur the error may be large. Consequently it is best to prepare a number of standards and use the one nearest the unknown color for comparison.

A typical experiment used in trying out the method is given here.

A solution containing the following in 1 cc. was employed:

	mgm.
Aluminium sulphate	0.01
Ferric chloride	0.5
Calcium chloride	0.05
Mono potassium phosphate	0.05
Magnesium chloride	0.05
Sodium chloride	3

This approaches, with the exception of the aluminium, the composition of the ash of blood. Five cubic centimeters of this mixture were taken, shaken out with ether as described. Various quantities were used and compared against a standard containing 0.01 mgm. aluminium, set at 20 mm. The results follow:

	AMOUNT TAKEN	READING	CALCULATED	PRESENT	ERROR
	cc.				*per cent*
Standard	1	19.9	0.010	0.01	0
mixture	1.4	17.4	0.0115	0.0116	<1
	1.2	19.9	0.010	0.01	0
	1.4	17.4	0.0115	0.0116	<1
	1	19.9	0.0084	0.0083	

The blank in this case was too different in color to be compared, being a greenish yellow as against the pinkish yellow of the standards. This type of experiment was tried again and again with various proportions of the various substances. In this way it was determined that the inorganic ions occurring in the largest quantities in the ash of blood do not interfere with this method.

SUMMARY

A sensitive method for the estimation of small quantities of aluminium in biological material is described. When the precautions detailed are observed the method is highly satisfactory.

BIBLIOGRAPHY

(1) Smith, E. E. Aluminium compounds in food. Hoeber, New York, 1928.
(2) Stoklasa, J. Über die Verbreitung des Aluminiums in der Natur. Gustav Fischer, Jena, 1922.
(3) Fillinger, F. V. Zeitschr. f. Nahr. Genussmittel, 1908, xvi, 232.
(4) Utz. Zeitschr. angew. chem., 1919, xxxii, 345.
(5) Mirasol, J. J. Soil Science, 1920, x, 153. (N. B. A thorough review of aluminium in relation to plants and agriculture.)
(6) Carr, R. H. and P. H. Brewer. Journ. Ind. Eng. Chem., 1924, xv, 634.
(7) R. I. Agric. Exp. Sta. Bull., 1923, cxciv.
(8) Hartwell, B. L. and F. R. Pember. Soil Science, 1918, vi, 259.
(9) Hebert, A. Journ. Physiol. path. gen., 1907, ix, 751.
(10) Hebert, A. Bull. de Soc. Chim., 1907, iv, 1026.
(11) Cook, A. N. and S. Elliott. Journ. Ind. Eng. Chem., 1916, viii, 503.
(12) Siem, P. Dissertation, Dorpat, 1886.
(13) Hanzlik, P. J. and E. Presho. Journ. Pharm. Exper. Therap., 1923, xxi, 145.
(14) Myers, V. C. and J. W. Mull. Journ. Biol. Chem., 1928, lxxviii, 605.
Myers, V. C. and D. B. Morrison. Ibid., 1928, lxxviii, 615.
(15) McCollum, E. V., O. S. Rask and J. E. Becker. Journ. Biol. Chem., 1928, lxxvii, 753.
(16) Wright, N. C. and J. Papish. Science, 1929, lxix, 78.
(16a) Kahlenberg, L. and J. O. Closs. Science, 1929, lxix, 186.
(17) Atack, F. W. Journ. Soc. Chem. Ind., 1915, xxxiv, 936.
(18) Hatfield, W. D. Journ. Ind. Eng. Chem., 1923, xvi, 233.
(19) Wolff, L. K., N. J. M. Vorstman and P. Schoenmaker. Chem. Weekblad., 1923, xx, 193.

(20) PHILIP, J. C. AND A. BRAMLEY. Journ. Chem. Soc., 1913, ciii, 795; Proc. Chem. Soc., 1913, xxix, 123.
(21) TREADWELL, F. P. AND W. T. HALL. Analytical chemistry. 4th ed., 1916.
(22) CLOVER, A. M. Journ. Amer. Chem. Soc., 1922, xliv, 1107.
(23) ZECHNER, L. AND F. WISCHO. Pharm. Monatshefte, 1924, v, 133, 465.
(24) SALM, B. Zeitschr. physik. Chem., 1906, lvii, 471.
(25) MCILVAINE, T. C. Journ. Biol. Chem., 1921, xlix, 183.

STUDIES IN THE METABOLISM OF ALUMINIUM

II. Absorption and Deposition of Aluminium in the Dog[1]

FRANK P. UNDERHILL AND F. I. PETERMAN

From the Department of Pharmacology and Toxicology, Yale University, New Haven, Conn.

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The fact that the majority of investigators have not found aluminium in the blood and tissues of animals after a diet containing appreciable amounts of it (1), has in the main been attributed to the formation, in the gastro-intestinal tract, of insoluble compounds, that is, aluminium hydroxide, or phosphates, or aluminium-protein complexes. That insoluble compounds may be formed during the baking of aluminium-containing breads has also been suggested.

It has been demonstrated that some of the aluminium occurring naturally in foods is soluble in aqueous solutions (2). It has been shown repeatedly that a large percentage of the aluminium in bread made with alum baking powders is soluble in gastric juice (1). It is unknown whether aluminium forms some complex protein compound in bread. At any rate after exposure to gastric juice, either *in vitro* or *in vivo*, the resulting compound is dialyzable, and is presumably the chloride.

Information as to the exact degree of alkalinity of the intestinal tract is lacking but the available evidence seems to indicate that the pH does not vary widely from pH 7.0 (3) (4) (5).

The pH for the precipitation of aluminium hydroxide varies, of course, with the constituents of the solution. In pure solutions it begins to precipitate at pH 4.1 (6). But the hydroxide is truly amphoteric, the soluble aluminates forming promptly. Aluminium phosphate begins to precipitate, under various conditions at pH 3.9 to 5.5 (7) (8) (9). It starts to redissolve at pH 8.6.

While it is injudicious to hazard a guess as to what happens in an exceedingly complex mixture it seems probable that aluminium compounds may be largely soluble in the intestinal tract.

[1] Constructed, in part, from a dissertation presented to Yale University in June, 1925, by F. I. Peterman in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Summarizing we may suppose that aluminium occurring naturally in foods, or added for leavening purposes, is soluble to a certain extent in both stomach and intestinal tract. Since it is soluble, we must expect that it will be absorbed into the body.

The purpose of the present investigation was to obtain information relative to the following problems:

1. The aluminium content of the blood of dogs
   - *a*, in the normal fasting condition
   - *b*, on a diet to which the aluminium has been added
   - *c*, at various times after a single feeding of food to which aluminium has been added
   - *d*, during and after a dietary of aluminium containing foods for periods of
     - (1) 1 week
     - (2) 4 weeks
     - (3) 12 weeks
   - *e*, during and after an ordinary dietary for periods corresponding to *d*
2. The aluminium content of the tissues of these dogs.

GENERAL METHODS. The experimental animals, in good nutritive condition, were of unknown origin but were full grown and as near as one may judge did not exceed five years in age. Kept in separate cages in the animal house they were fed ground lean beef, aluminium-containing biscuits and butter. Control animals were fed yeast bread instead of biscuits. The dogs were given as much as they would eat, but only one feeding per day.

Two kinds of baking powder were used in the preparation of the biscuits, which for the sake of brevity will be designated as "straight alum" and "alum-phosphate" biscuits.

As it was not possible to purchase a straight alum baking powder on the local market one was prepared in the laboratory, and had the following composition:

Sodium aluminium sulphate, calcined	25 grams
Sodium bicarbonate, dry	26 grams
Corn starch	49 grams

This powder was used in biscuits as follows:

Flour	8100 grams
Salt	75 grams
Baking powder	540 grams
Butter	300 grams
Water	q.s.

The "alum-phosphate" powder used was a well-known brand, bought on the market, and according to the label its composition was:

Mono calcium phosphate	15.67 per cent
Sodium aluminium sulphate	12.33 per cent
Sodium bicarbonate	25.00 per cent
Starch	47.00 per cent

TABLE 1

The aluminium content of blood of fasting dogs

Al as mgm. per 100 cc. of blood

DAY	DOG 1	DOG 2	DOG 3	DOG 4	DOG 5	DOG 6	AVERAGE
1	0.28	0.35	0.28	0.32	0.17	0.17	0.23
3	0.29	0.32	0.28	0.27	0.17	0.17	
5	0.28	0.28	0.27	0.17	0.16	0.18	
7	0.28	0.27	0.27	0.16	0.16	0.16	
Average....	0.28	0.30	0.27	0.23	0.16	0.17	

TABLE 1A

Aluminium content of tissues of dogs fasted one week

Al as mgm. per 100 grams of wet tissue

DOG NUMBER	WEIGHT	SEX	LIVER	BILE	KIDNEY	BRAIN	MUSCLE	HEART	SPLEEN	ADRENAL	OVARY	TESTICLE	THYROID
1	17.3	F.	0.66	1.80	0.57	1.22	0.138	0.213	1.36	1.01	None		5.5
2	16.0	M.	0.69	2.01	0.74	1.10	0.135	0.222	1.41	None		None	6.0
3	18.6	M.	0.89	1.85	0.84	1.05	0.135	0.191	2.06	2.49		None	7.4
4	12.0	F.	0.80	2.84	0.94	1.08	0.200	None	2.04	None	None		11.4
5	15.5	M.	0.90	1.39	0.66	1.27	0.131	None	1.67	None		—	4.7
6	14.0	F.	0.71	2.16	0.56	1.17	None	0.937	2.27	None	None		5.0
7	16.4	F.	0.90	2.99	1.45	0.96	0.125	0.270	2.29	1.69	None		8.2
8	14.8	F.	0.75	1.74	0.90	1.18	0.142	None	1.43	None	None		8.2
9	3.6	M.	1.41	1.00	0.84	1.31	0.127	0.252	2.37	None		Trace	10.3
10	8.9	M.	0.95	2.19	1.20	1.21	0.129	Trace	2.38	None		Trace	12.4
11	9.0	F.	1.33	0.92	0.89	2.17	None	None	1.32	1.01	None		6.1
12	15.0	M.	1.16		0.72			0.220	1.49	None		None	2.3
Average....			0.94	1.90	0.86	1.25	0.114	0.192	1.84	0.51	None	0.000	7.25

Biscuits were made approximately according to the directions on the can, and contained:

Flour	8750 grams
Baking powder	500 grams
Salt	80 grams
Butter	300 grams
Water	q.s.

The biscuit dough was mixed and baked much as it is by the housewife. After baking it was broken into pieces, thoroughly dried and then ground to a coarse meal. The dogs would eat a moist mixture of hashed meat and biscuit meal, but if the biscuit were given in pieces they would eat the meat and leave the biscuit. Biscuit dried and ground kept well without

TABLE 2

Aluminium content of blood of dogs fed on control diet for one week

Al as mgm. per 100 cc. of blood

DAY	DOG 19	DOG 20	DOG 21	DOG 22	DOG 23	DOG 24	AVERAGE
1		0.45	0.37	0.31	0.29	0.27	0.36
5	0.35	0.48	0.44	0.31	0.38	0.30	
8	0.36	0.48	0.40	0.37	0.37	0.31	
Average....	0.35	0.47	0.40	0.33	0.34	0.29	

TABLE 2A

Aluminium content of tissues of dogs after one week's feeding of "normal diet"

Al as mgm. per 100 grams of wet tissue

DOG NUMBER	WEIGHT	SEX	LIVER	BILE	KIDNEY	BRAIN	MUSCLE	HEART	SPLEEN	ADRENAL	OVARY	TESTICLE	THYROID
13	10.6	F.	0.77	—	1.56	1.64	—	None	2.39	None	None		2.7
14	14.0	F.	0.56	1.56	1.40	1.95	None	0.227	2.09	None	None		8.5
15	7.4	F.	0.93	1.62	1.40	2.14	None	Trace	2.03	None	—		7.4
16	5.6	F.	0.74	2.77	0.96	1.33	None	0.513	1.97	None	None		1.5
17	12.0	F.	0.67	—	1.58	—	None	0.608	2.24	None	—		1.6
18	12.6	F.	0.56	2.06	0.86	—	None	0.465	1.74	None	None		7.3
19	14.6	F.	1.24	1.92	1.09	1.06	None	Trace	1.52	None	None		8.7
20	14.2	M.	0.31	2.10	1.47	1.14	None	0.258	2.11	None		None	5.1
21	10.0	F.	0.31	1.92	0.64	0.96	0.065	0.144	2.03	None	None		4.0
22	15.5	M.	0.36	1.50	0.51	1.42	Trace	None	1.88	Trace		None	5.2
23	14.7	F.	0.41	1.94	0.80	1.78	Trace	Trace	2.07	Trace	None		11.0
24	23.4	F.	0.63	1.19	1.21	1.08	None	None	2.05	Trace	None		4.5
Average...			0.66	1.86	1.12	1.45	0.000	0.184	2.01	0.000	0.000	0.000	5.5

becoming mouldy or sour. A large "batch" could thus be made at one time.

Inasmuch as in these experiments our attention was directed mainly to the question of aluminium absorption and distribution in the tissues no attempt was made to determine exactly the quantities of aluminium ingested. However, from calculation it is certain that for the straight alum

TABLE 3

Aluminium content of the blood of dogs after a single meal of aluminium-containing food

Dogs 25 to 31 inclusive and 38 and 39—straight alum biscuit.
All others—alum-phosphate biscuit. (Al as mgm. per 100 cc. of blood.)

TIME	DOG 25	DOG 26	DOG 27	DOG 28	DOG 29	DOG 30	DOG 31	DOG 32	DOG 33	DOG 34	DOG 35	DOG 36	DOG 37	DOG 38	DOG 39	DOG 40	DOG 41	DOG 42	DOG 43
2 days previous		0.82		Fasting			Ashed in porcelain	Fasting							Fasting				
1 day previous				1.10	1.06	Fasting				0.30	0.29			Fasting	0.62	Fasting, trace	0.000	0.000	0.058
Same day					1.04			0.88	0.77			0.38	0.58	0.77					
Fed																			
½ hour	0.55	0.92	0.54	1.87	1.06	1.48		0.36	0.78	0.36	0.22	0.39	0.56	0.55	0.73				
1 hour	0.38	1.04	0.55					0.78	0.78			0.52		0.51	0.54			0.000	0.099
2 hours	0.32									0.36	0.70		0.40			0.051	0.000		
3 hours		0.78	0.74					0.56	0.56			0.37		0.73	0.54			0.000	0.057
4 hours	0.86			1.19	0.43	0.92				0.28	0.53		0.65			0.048	0.000		
5 hours		0.96	0.72					0.74	0.84			0.72		0.54	0.53			0.000	0.029
6 hours	0.71				0.41	1.90				0.29	0.53		0.39			Trace	0.000		
7 hours			0.56					0.67	0.51			0.72		0.54	0.53			0.000	0.057
8 hours	1.06			1.04	0.40	0.97				0.74	0.22		0.39			Trace	0.000		
9 hours		1.17	1.24					0.72	0.91			0.22		0.73	0.45			0.000	0.043
10 hours				0.95	0.36	0.76				0.28	0.22		0.38			Trace	0.000		
24 hours		1.02	0.54	0.89	0.73	0.37		0.61	0.73	0.22	0.15	0.38	0.38	0.37	0.46	Trace	0.000	0.000	Trace
Average	0.64	0.95	0.67	1.16	0.68	1.06		0.66	0.73	0.35	0.35	0.46	0.46	0.59	0.55		0.000	0.000	0.049

biscuits the quantity of aluminium taken in was never less than 100 mgm. daily, and in most instances was much greater. The figures for the alum phosphate biscuits are probably somewhat lower than this.

Blood was drawn at specified intervals from the jugular vein into a Luer syringe containing aluminium-free oxalate. Enough was taken for an ash sample (usually about 10–20 cc.). When the animals were killed they were bled as completely as possible under ether anesthesia. The organs were removed carefully to avoid accidental contamination. The

TABLE 3A

Aluminium content of tissues of dogs after a single meal of aluminium-containing food

Al as mgm. per 100 grams of wet tissue

DOG NUMBER	WEIGHT	SEX	LIVER	BILE	KIDNEY	BRAIN	MUSCLE	HEART	SPLEEN	ADRENAL	OVARY	TESTICLE	THYROID
25	19.0	M.	0.84	1.99	—	1.34	None	0.487	1.23	0.48		—	2.6
26	15.0	M.	0.93	1.64	1.66	2.16	None	0.115	1.35	None		None	4.2
27	16.4	M.	0.50	1.91	0.53	2.07	0.032	0.136	1.19	—		None	2.8
28	16.8	F.	0.44	1.98	0.58	1.61	0.124	0.500	2.07	None	None		5.3
29	19.8	M.	—	1.75	1.21	1.18	0.065	0.132	2.07	None		None	5.2
30	11.8	F.	0.64	6.13	0.52	2.04	Trace	0.278	2.00	None	None		16.5
31	7.0	M.	0.87	2.49	0.98	2.00	0.114	0.283	1.99	None		None	3.4
32	15.6	F.	0.49	2.24	0.85	2.27	Trace	None	1.66	None	None		6.9
33	13.2	F.	0.84	2.63	0.48	1.56	None	0.280	2.52	None	None		3.2
34	14.0	M.	0.32	2.55	0.41	1.34	Trace	0.377	2.05	None		None	5.3
35	14.0	M.	0.61	2.29	0.62	1.78	Trace	0.570	2.65	None		None	—
36	22.0	F.	0.65	2.32	0.55	1.84	Trace	0.258	2.68	None	Trace		6.2
37	17.9	F.	0.49	3.38	0.59	1.20	None	0.365	2.66	None	None		5.2
38	20.0	M.	0.42	2.48	1.11	1.11	0.193	None	2.77	None		0.147	4.1
39	21.7	M.	0.42	1.68	0.67	1.14	0.125	0.206	2.82	None		0.167	4.4
Average.....			0.60	2.50	0.76	1.64	0.040	0.265	2.16	0.034	0.000	0.039	5.3

organs reserved were kidneys, liver, gall bladder, spleen, adrenals, ovaries, testes, brain, muscle and heart.

Blood samples were measured and dried immediately after drawing. Every care was taken to avoid accidental contamination of material. Analyses were made according to the method described in the preceding paper (10).

EXPERIMENTS. *Group I—Aluminium content of blood and tissues of fasting dogs.* There were twelve dogs in this series. They were starved for seven days. Distilled water was allowed freely. Blood samples were taken on the morning of the first, third, fifth and seventh day of starvation. After the last sample had been taken the dogs were killed according to the

TABLE 4

The aluminium content of the blood of dogs fed for one week on aluminium-containing food

Al as mgm. per 100 cc. of blood

DAY	HOUR AFTER FEEDING	STRAIGHT ALUM BISCUIT			ALUM-PHOSPHATE BISCUIT			CONTROL—YEAST BREAD	
		Dog 44	Dog 45	Dog 46	Dog 47	Dog 48	Dog 49	Dog 50	Dog 51
1	1	0.81			0.49			0.44	
	2		0.37			0.73			0.49
	3			0.39			0.36		
2	4	0.35			0.43			0.37	
	5		0.37			0.35			0.52
	6			0.38			0.38		
3	7	0.73			0.60			0.51	
	8		0.44			0.35			0.52
	9			0.38			0.45		
4	10	0.75			0.70			0.43	
	11		0.38			0.55			0.51
	12			0.39			0.52		
5	13	0.37			0.38			0.45	
	14		0.38			0.36			0.51
	15			0.39			0.52		
6	16	0.84			0.71			0.46	
	17		0.57			0.98			0.51
	18			0.37			0.57		
7	19	1.09			1.04			0.66	
	20								0.54
	21			1.47					
	Killed								
	HOURS AFTER LAST FEEDING								
	32	0.32	0.29	0.39					
	33	0.37				0.29			
	34				0.52		0.38		
	35							0.33	0.28
Average.....		0.63	0.39	0.52	0.60	0.51	0.45	0.44	0.46

procedure already described. The blood samples from the first six were ashed in porcelain and found to give doubtful results. These were discarded. The samples from the other six were ashed in silica. The organs of all were analysed as usual; the results for these are given here. All determinations were done in duplicate. In tables 1 and 1A are detailed the results for this group.

Group II—Aluminium of blood and tissues of dogs on a normal diet. Twelve dogs were kept without food for two days, then fed daily on a diet which was considered normal with respect to possible aluminium content. It consisted of hashed beef, yeast bread, and butter, and is herein

TABLE 4A

Aluminium content of tissues of dogs fed on aluminium-containing food for one week

Al as mgm. per 100 grams of wet tissue

DOG NUMBER	WEIGHT	SEX	LIVER	BILE	KIDNEY	BRAIN	MUSCLE	HEART	SPLEEN	ADRENAL	OVARY	TESTICLE	THYROID
44	13.2	F.	0.64	2.22	0.44	1.34	0.026	1.62	1.95	1.02	None		9.2
45	17.2	M.	0.82	2.34	0.44	2.01	Trace	0.195	1.99	0.78		0.000	5.3
46	12.4	M.	0.68	2.31	0.53	1.82	0.111	0.188	2.23	None		0.000	2.9
47	10.3	F.	1.43	2.05	0.57	1.58	Trace	0.344	1.57	None	None		5.7
48	11.0	F.	0.98	1.78	0.55	1.25	None	0.353	1.28	None	None		9.3
49	10.6	M.	0.51	2.96	0.43	1.76	0.121	0.300	2.12	None		0.138	4.2
Average.....			0.84	2.27	0.50	1.63	0.043	0.500	1.85	0.30	None	0.046	6.1
50	9.2	F.	0.47	1.84	0.70	1.32	None	0.293	2.39	None	None		12.8
51	10.0	F.	0.42	2.20	0.80	1.31	Trace	Trace	2.51	None	None		8.1
Average (control)..			0.44	2.02	0.75	1.31	0.000	0.147	2.45	0.000	0.000		10.4

designated the "control diet." The first six were bled only on the last day of the feeding period. The bloods were analysed by the trichloracetic acid filtrate method which was found to give unreliable results, hence are not included. The other six were bled on the first, fifth and eighth days, at various times after feeding, and were killed on the eighth day. All tissues were ashed in silica. The results may be seen in tables 2 and 2A.

Group III—Aluminium content of blood of dogs after a single meal of aluminium-containing food. This series consisted of nineteen dogs. They were fasted for one or two days, then given a single feeding of alum-phosphate or straight alum biscuit mixed with meat. Blood was drawn at definite intervals after feeding. Blood samples of dog 31 were ashed in

TABLE 5

Influence of four weeks' feeding of aluminium-containing food on the aluminium content of the blood

Al in mgm. per 100 cc. of blood

WEEK	HOUR AFTER FEEDING	STRAIGHT ALUM BISCUIT			ALUM-PHOSPHATE BISCUIT			CONTROL—YEAST BREAD	
		Dog 52	Dog 53	Dog 54	Dog 55	Dog 56	Dog 57	Dog 58	Dog 59
1	2	0.53			0.61			0.81	
	4		0.78			0.55			0.20
	6			0.93			0.50		
2	2	0.20			0.53			0.35	
	4		0.53						0.20
	6			0.63			0.32		
3	2	0.23			0.55			0.27	
	4		0.33			0.26			0.27
	6			0.47			0.27		
4	2	0.27			0.60			0.33	
	4		0.26			0.69			0.16
	6			0.28			0.28		
	Killed								
	HOURS AFTER LAST FEEDING								
	7	0.33	0.46		0.56	0.86			
	9			0.46			0.28		
	39							0.22	0.16
Average.....		0.31	0.47	0.55	0.57	0.59	0.33	0.40	0.20

Weekly averages—blood

WEEK	STRAIGHT ALUM	ALUM-PHOSPHATE	CONTROL
1	0.75	0.55	0.51
2	0.46	0.42	0.28
3	0.34	0.36	0.27
4	0.27	0.52	0.25
At death	0.41	0.56	0.19
Grand average...	0.45	0.48	0.30
	0.47		

porcelain, the results of which were discarded. The animals were killed after twenty-four hours. Tissues of fifteen of them have been analysed. The results are shown in tables 3 and 3A.

Group IV—Aluminium content of blood and tissues of dogs maintained upon aluminium-containing food for a period of one week. Eight dogs were fed for one week on a diet containing straight alum or alum-phosphate biscuit or yeast bread. Blood samples were taken each day at definite intervals after feeding. Since there was some indication in group III that greater absorption occurred in the later hours after feeding, it was planned to follow the course of absorption through the whole twenty-four hour cycle. Thus information might be gained concerning the time factor as

TABLE 5A

Aluminium content of tissues of dogs fed on aluminium-containing food for one month

Al as mgm. per 100 grams wet tissue

DOG NUMBER	WEIGHT	SEX	LIVER	BILE	KIDNEY	BRAIN	MUSCLE	HEART	SPLEEN	ADRENAL	OVARY	TESTICLE	THYROID
52	11.0	F.	0.49	2.14	0.98	1.58	0.188	0.063	2.04	None	None		6.4
53	11.1	F.	0.52	2.07	0.76	0.97	0.117	None	1.35	None	None		3.1
54	8.6	M.	0.74	1.68	0.51	0.80	0.112	0.104	2.11	None		0.399	10.2
55	11.6	F.	0.50	1.42	0.71	1.48	None	0.508	1.82	None	None		6.0
56	13.0	M.	0.88	1.52	0.42	1.20	None	0.532	2.67	None		0.348	2.9
57	8.0	M.	1.35	1.70	0.48	1.17	0.153	0.528	2.59	None		0.263	2.5
Average.....			0.73	1.75	0.65	1.20	0.095	0.289	2.12	None	None	0.336	5.2
58	13.0	F.	0.66	1.32	0.79	1.20	0.028	0.139	1.65	None	None		4.6
59	16.0	F.	0.68	1.48	0.51	1.11	Trace	0.217	1.98	None	None		3.4
Average (control)..			0.67	1.40	0.65	1.15	0.014	0.178	1.82	None	None		4.0

well as the height to which the aluminium in the blood might possibly rise. For results see tables 4 and 4A.

Group V—Aluminium content of blood and tissues of dogs maintained upon aluminium-containing food for a period of four weeks. Eight dogs were fed for four weeks upon a diet identical with that of group IV. Blood samples were taken on the seventh day of each week at a definite time after the meal. Tables 5 and 5A show the results obtained.

Group VI—Aluminium content of blood and tissues of dogs maintained upon aluminium-containing food for a period of twelve weeks. Eight dogs were fed for twelve weeks. Blood samples were drawn at definite intervals

TABLE 6

The aluminium content of blood of dogs fed aluminium-containing food for twelve weeks

Al as mgm. per 100 cc. of blood

WEEK	HOUR AFTER MEAL	STRAIGHT ALUM BISCUIT			ALUM-PHOSPHATE BISCUIT			CONTROL—YEAST BREAD	
		Dog 60	Dog 61	Dog 62	Dog 63	Dog 64	Dog 65	Dog 66	Dog 67
1	2	0.65						0.36	
	4		0.27			0.32			0.17
	6								
2	6	0.22						0.22	
	8		0.38			0.40			0.41
	10			0.32					
3	10	0.40			0.65				
	12		0.39			0.39			0.28
	14			0.43					
4	2	0.55			0.54			0.26	
	4		0.39			0.33			0.59
	6			0.33			0.32		
5	6	0.38			0.39			0.28	
	8		0.32			0.27			0.40
	10						0.39		
6	10	0.41			0.55			0.28	
	12		0.33			0.27			0.39
	14			0.33			0.36		
7	2	0.57			0.60			0.16	
	4		0.56			0.27			0.40
	6			0.42			0.28		
8	6	0.62			0.51			0.22	
	8		0.38			0.22			0.33
	10			0.44			0.38		
9	10	0.39			0.62			0.21	
	12		0.32			0.46			0.38
	14			0.43			0.39		
10	2	0.16			0.55			0.21	
	4		0.38			0.21			0.36
	6			0.39			0.28		
11	6	0.29			0.27			0.18	
	8		0.31			0.33			0.38
	10			0.44			0.28		

TABLE 6—*Concluded*

WEEK	HOUR AFTER MEAL	STRAIGHT ALUM BISCUIT			ALUM-PHOSPHATE BISCUIT			CONTROL—YEAST BREAD	
		Dog 60	Dog 61	Dog 62	Dog 63	Dog 64	Dog 65	Dog 66	Dog 67
12	10	0.27			0.27			0.22	
	12		0.31			0.21			0.25
	14			0.33			0.27		
Killed	25								
13	2				0.27				
	4								
	6			0.41			0.28		0.16
Killed	7			0.41					0.17
14	6				0.27				
	8								
	10						0.27		
Killed	23				0.24		0.32		
Average.....		0.41	0.35	0.39	0.44	0.30	0.32	0.24	0.33
		0.38			0.35			0.28	

TABLE 6A

Aluminium content of tissues of dogs kept for twelve weeks on an aluminium-containing diet

Al as mgm. per 100 grams wet tissue

DOG NUMBER	WEIGHT	SEX	LIVER	BILE	KIDNEY	BRAIN	MUSCLE	HEART	SPLEEN	ADRENAL	OVARY	TESTICLE	THYROID
60	8.9	F.	0.73	1.98	0.59	1.09	0.058	0.210	2.51	0.85	None		7.9
61	9.0	F.	1.05	2.56	0.52	1.55	0.028	0.166	2.47	1.31	None		7.2
62	10.3	F.	0.75	1.38	0.50	1.04	Trace	0.489	2.64	Trace	None		5.8
63	11.1	M.	0.70	2.83	0.60	2.52	None	None	2.54	0.25		—	4.6
64	15.0	M.	0.77	1.22	0.55	1.09	None	0.207	2.13	None		—	2.9
65	12.6	M.	0.97	1.82	0.48	4.19	None	0.065	2.20	None		None	8.2
Average....			0.83	1.95	0.54	1.91	0.014	0.189	2.41	0.40	None	None	6.1
66	15.6	F.	0.84	1.61	0.55	1.45	Trace	0.100	2.14	Trace	None		5.1
67	16.2	F.	0.63	1.26	0.61	1.11	0.010	0.034	2.08	0.33	None		2.6
Average (control).			0.74	1.44	0.58	1.28	0.005	0.067	2.11	0.16	None		3.8

after the meal of the seventh day. Food was given at a regular time each day. The general condition of the animals was observed frequently. On the whole they ate well and remained in good nutritive condition. Three had to be replaced by others after the beginning of the experiment, one because it would not eat, two because they were vicious. The results are recorded in tables 6 and 6A.

In table 7 may be found average results for all groups of animals thus far considered.

TABLE 7

Summary of averages of determination of all groups

Al as mgm. per 100 grams wet tissue

PROCEDURE	NUMBER	LIVER	BILE	KIDNEY	BRAIN	MUSCLE	HEART	SPLEEN	ADRENAL	OVARY	TESTICLE	THYROID
1 wk. starving	12	0.94	1.90	0.84	1.25	0.114	0.192	1.84	0.51	None	0.000	7.25
1 wk. meat and bread	12	0.66	1.86	1.12	1.45	Trace	0.184	2.01	None	None	0.000	5.50
Single feeding	15	0.60	2.50	0.76	1.64	0.040	0.265	2.16	0.034	None	0.039	5.30
1 wk. feeding	6	0.84	2.27	0.50	1.63	0.043	0.500	1.85	0.300	None	None	6.10
1 wk. control	2	0.44	2.02	0.75	1.31	Trace	0.147	2.45	None	None		10.40
1 mo. feeding	6	0.73	1.75	0.65	1.20	0.095	0.289	2.12	None	None	0.336	5.20
1 mo. control	2	0.67	1.40	0.65	1.15	0.014	0.178	1.82	None	None		4.00
3 mo. feeding	6	0.83	1.95	0.54	1.19	0.014	0.189	2.41	0.400	None	0.000	6.10
3 mo. control	2	0.74	1.44	0.58	1.28	0.005	0.067	2.11	0.016	None		3.80

DISCUSSION OF RESULTS. *The aluminium content of blood and tissues of fasting dogs.* So far as one may glean from the literature, reliable data are lacking relative to the presence of aluminium in the blood of fasting animals. It has been stated at times that aluminium is a constant constituent of the tissues of animals and man, but equally positive statements have denied this (vide, e.g., McCollum et al., 11). From our own work with fasting dogs it is apparent (see table 1) that the blood of dogs fasted for seven days contains appreciable quantities of aluminium. The extreme limits of the concentration were 0.16 to 0.35 mgm. of aluminium per 100 cc. of blood, the average being 0.23 mgm. The delicacy of the method is such that these quantities must be regarded as significant. They cannot be laid to errors inherent in the method since experience has shown that the limit of error is a figure lying in the third decimal place, approximately 0.001, so small it may be disregarded.

The quantity of aluminium in the blood does not tend to diminish appreciably during this period of fasting. This must indicate previous storage in the body. In order that the amount in the blood remain at a

fairly constant level as fasting progresses there must be deposited somewhere in the body much larger quantities.

From an examination of table 1A it is evident that the notable places of storage for aluminium are the liver, kidney, brain and spleen.

The *liver* contains, on an average 0.94 mgm. per 100 grams of tissue, more than four times as much aluminium per unit as blood. The metal is, possibly, in the starving condition, on its way to excretion. The aluminium is released slowly, perhaps because the body is not capable of excreting it rapidly, or because the body has some use for it. Just how long the starvation would have to be prolonged in order to render the blood aluminium-free has not been determined, but it would seem, from an examination of the figures, that it might take a number of weeks. At the beginning of starvation the quantity in the organs may perhaps be assumed to have been larger.

The *kidney* is also a locus of storage, containing slightly less than the liver, and nearly four times (average) as much as the blood. That the kidney is also a channel for excretion of aluminium is to be inferred from the definite amounts found there. On the other hand, various investigators have failed to find aluminium in the urine of animals and man fed with aluminium containing foods. It is probable, however, that with a method sufficiently delicate, small quantities would have been found. In a subsequent paper evidence of the urinary excretion of aluminium will be presented.

That the kidney cannot be the most important excretory path is clear from the amounts found in the *bile*. Here, comparatively large quantities, more than eight times that found in the blood, are present, showing that aluminium is excreted principally through the bile, and that the liver of the fasting animal must be the organ chiefly concerned with picking out and excreting this metal. It is apparent that much of the aluminium excreted in this way may be reabsorbed.

In this group the *spleen* contains by far the greatest store, per unit, of aluminium, more than eight times the amount found in the blood. The large amount here cannot be attributed to the blood contained in the organ, especially as the animals were bled to death and the spleen was invariably found at autopsy to be quite contracted and hard. However, as this organ is very small as compared with the liver, its actual importance as a storage place for aluminium must not be over emphasized. But the fact that per unit it holds nearly twice as much as liver is interesting, however obscure the reason.

The *brain* also holds a larger store of aluminium per 100 grams of tissue than the liver and more than five and one-half times per unit as blood.

The *heart* and *voluntary muscle* contain relatively small amounts of aluminium. But when we consider the large mass of muscular tissue, as

compared with the spleen, for instance, it is evident that the total aluminium stored in the muscle mass may reach an important figure.

The *adrenals* contain very little aluminium, the *ovary* none, the *testicle* only a trace, if any. The large amount in the *thyroid* is very striking. It must be pointed out that with a tissue as small as adrenal, ovary or thyroid the error is multiplied greatly in the final calculation. But this is not the most important factor since the average for the aluminium actually present in the thyroid glands was 0.078 mgm., a significant figure. In the adrenals often no aluminium is found but there is always an appreciable amount present in the thyroid.

From these observations one is forced to conclude that aluminium occurs in the blood and tissues of animals maintained in a fasting conditions for a period of seven days. The spleen, brain, liver and kidney hold relatively large amounts, while the bile is richest of all in aluminium.

In view of these facts one must, then, consider aluminium as a constant constituent of the adult animal body. The bile may be regarded as the chief path of excretion of aluminium.

The aluminium content of the blood and tissues of dogs maintained on an ordinary (control) diet. When one turns to an examination of the results for the second group of dogs (table 2), those on a diet considered "normal," in that no aluminium had been added to it, one finds a slightly different state of affairs. The aluminium in the blood is, on the average, 0.13 mgm. per 100 cc. higher than in the fasting dogs, while the liver averages are lower by 0.28 mgm., the bile by 0.04 mgm., the kidney higher by 0.28 mgm., the brain by 0.20 mgm. and the spleen by 0.17 mgm. These differences, except for the bile, are significant.

The slightly higher value for the *blood* points either to absorption from food, or, in view of the smaller quantity found in the liver (table 2A), to an augmented release of the metal from the liver into the blood stream. The increase in aluminium may well come from the food ingested, since it has been shown that practically all of food substances may contain aluminium (12). Flour is particularly rich in this element. Whether the aluminium in foods is due to its natural occurrence or whether it may be regarded as a contamination cannot be decided at present. It is probable that with some foods both alternatives are correct.

In spite of the increase in the blood there is not a corresponding increase in the *bile*. The bile contains practically the same amount of aluminium as that of the fasting dogs. The ratio, however, between the content of the bile and liver is higher than for the fasting dogs, pointing, perhaps, to a more rapid excretion.

The quantity of aluminium found in the *kidney* is greater than that found in the kidney of the fasting dogs, and about twice as much as that found in the livers of the same group (II). This may mean increased

storage. Or it may merely be the result of the exposure of the kidney tissue to higher concentrations of aluminium in the blood. Not only that, but in view of the nutritional condition of the two groups of dogs, one might expect differences in the handling of any particular substance, so that in this case, the kidney may actually be more active in excreting the aluminium than it is in the starving animals.

That the *livers* of well fed animals may contain less aluminium than livers of starving ones is demonstrated. The explanation undoubtedly lies in the differences of the state of nutrition in the two groups. It may be possible that in the fasting animal changes in the composition of the liver have occurred which cause such a liver to yield an apparently higher content of aluminium than the liver of well fed animals, a liver which has not undergone a similar alteration in composition. It is interesting to note that the moisture content of the livers of the two groups varied somewhat. For starving animals 1 gram of dried liver was equivalent to 3.28 grams of wet tissue, for the fed animals the ratio was 1:3.46, (averages) showing a difference in composition with regard to the water found there. We know that the liver, during starvation, loses large stores of fat and glycogen. Each cell is a system maintaining chemical equilibrium with its surroundings. The loss of glycogen and fat must result in a more unstable equilibrium which tends to be adjusted in some way. All the ions which can be held are probably held tenaciously. Before a correct interpretation of these results can be made, therefore, a study of the influence of fasting on liver composition should be made.

The average figures for the *brain* and *spleen* in this group are higher than for the starving group, seemingly indicating that the tissue acts as a storage place for aluminium, which is taken from the blood by these organs.

The differences in the other organs are not striking. Only a trace of aluminium is found in the *muscle*, none in the *adrenal* or *ovary*. The difference in the thyroid we cannot explain.

The conclusions which may be drawn from this series of experiments are these: that the aluminium content of the blood of animals which have been fed on a diet to which no aluminium has been added, is slightly higher than for starving animals. The tissues examined also contained aluminium. The ratios between the values for aluminium are different, and point to a difference in the handling of this metal in the two nutritional states. As in starving animals the bile is a most important factor in the elimination of aluminium.

An "ordinary diet" in the sense that the term is used here, may be a source for the aluminium found in the blood.

The aluminium content of blood and tissues of dogs after a single feeding of food containing added aluminium. This section of the investigation was

an attempt to determine when aluminium first appears in the blood after a meal containing considerable amounts of it, and the period of greatest absorption. By this means it was hoped to establish definite laws for aluminium absorption. When the results are examined, however (table 3), it is found that variations in these factors are so great that no definite laws for aluminium absorption may be formulated. It was also hoped to determine any difference between the body's treatment of aluminium from alum-phosphate and straight-alum biscuits.

An examination of the results obtained from this group of animals brings to light interesting information relative to the behavior of aluminium in the body. The average aluminium content of the blood is higher than in either of the two preceding groups. Considering only averages, there is evidence that aluminium is absorbed more readily from food very rich in it than from an ordinary diet.

When one examines the data critically, however, wide variations from the average are seen to occur. This has not been observed in the two preceding groups. Also, many times the pre-feeding level is higher than in the control groups. If one uses the pre-feeding or the one-half hour level for comparison it is seen that the aluminium in the blood increases markedly in dogs 25, 27, and 30, moderately in dogs 26, 33, 34, 35, 36 and 43, and actually decreases in dogs 29, 32, 38 and 39, and does not appear at all in dogs 41 and 42.

When there is an increase the interval before its appearance differs greatly. In dog 25, after an initial decrease there was a rise in the fourth hour, the maximum occurring in the eighth hour. In dog 27 there was a gradual, slight rise then a fall, followed by a large increase in the ninth hour. In dog 30 the highest level was found in the sixth hour, in dog 35 at the end of two hours.

It is evident that wide variations in the aluminium content of the blood may occur at different times following the ingestion of food containing comparatively large quantities of it. The celerity with which aluminium appears in the blood after such a meal depends upon many factors, especially on the condition of the alimentary tract. If digestion were sluggish absorption might be retarded. If the aluminium-containing food, mixed with fairly large quantities of gastric juice, passed quickly into the intestines absorption might be very rapid.

That individual animals vary widely in their response to any given treatment is well known and must always be borne in mind. Dog 35 is perhaps the most interesting individual of this group. Not a robust or active animal, it ate less than any of the others, yet there was a prompt increase in the aluminium of the blood. This shows that the amount absorbed is not dependent on the absolute quantity of aluminium contained in the food.

For all these animals the rise of aluminium in the blood is very transitory, any such increase tending to disappear rapidly.

What may occur between the bleeding periods, or between the tenth and the twenty-fourth hour when the animals were killed, is not apparent. It is possible that those dogs showing little or no absorption might have exhibited an increase in the later hours. Gies (13) remarks that "the amount of aluminium in the blood at any moment after the ingestion of aluminized food, is but a fraction of the quantity that may occur in the blood of the individual during a given day—larger quantities circulate throughout the body meanwhile, under such conditions."

The fact that the blood of the animals showing little absorption (dogs 26, 28, 32, 38, 39) is generally much richer in aluminium than the others, suggests that previous to the experiment their diets had contained considerable quantities of aluminium. This fact may also be interpreted to mean that the blood or indeed any of the tissues may become, as it were, saturated with a given metallic ion for the time being and be then unable to carry any further added increment. Such a condition has been suggested for arsenic and there is no apparent reason why this explanation would not hold for aluminium.

The response of the animals to straight alum biscuit does not differ materially from the response to alum-phosphate biscuit. While the average for blood is slightly higher it can hardly be regarded as evidence of greater absorption since the values both at the beginning and end are consistently higher.

The *livers* (see table 3A) in this group show definitely less aluminium than the fasting, and slightly less than the control group. This, when the higher figure in the *bile* is considered, points to increased activity of the liver in picking out and excreting large quantities. This explains, in part, why the aluminium does not attain an even higher concentration in the blood, and why a high concentration is not maintained long.

That the *kidney* contains less aluminium than do the kidneys of the other group suggests that the body employs the most efficient means for the excretion of large amounts of this metal.

The quantity of bile excreted by dogs has been determined (14). It varies from 2.9 to 36.4 grams per kilogram of body weight. For the average weight of this group, 16.2 K., that would be 36.9 to 589.7 grams. If we assume, for the sake of illustration, that the bile as it is excreted, contains as much aluminium as that found in the gall bladder twenty-four hour after feeding, it would mean an actual excretion of 0.93 to 14.6 mgm. of the metal for twenty-four hours (using the average figure for bile). While these figures are only approximate, they serve to illustrate the point that surprisingly large quantities of aluminium may be excreted in this way.

The average aluminium content of *brain* and *spleen* is slightly higher than for the previous groups, as one might expect, realizing that the tissues have been exposed to a blood richer in it. The changes in the other tissues are less remarkable.

From these experiments it is evident that aluminium is absorbed from food to which it has been added, but the quantity appearing in the blood does not reach a high level. The rise is transitory, the quantity tending to diminish rapidly. The aluminium residues in either type of biscuit, supposed to be aluminium phosphate in one case and aluminium hydroxide in the other, must be in part soluble, and the aluminium from either source is absorbed.

Aluminium is found in the most important organs of the body, in the brain and spleen of these animals in larger quantities than in controls, in the liver and kidney less. The bile is, however, richest in aluminium and must be considered a most important excretory path.

Aluminium content of blood and tissues of dogs maintained for one week on a diet to which aluminium had been added. Table 4 shows what was suggested before, that absorption tends to be greater in the later hours after the ingestion of aluminized foods. In dogs 44, 46 and 47 there are marked increases in the aluminium of the blood in the later hours of the last two days. In dogs 45, 48 and 49 the aluminium rises slightly. Even dog 50, a "control," shows the same tendency. While the averages here are not much higher than for the normal animals of this series or of group II, this relatively large increase in the sixth and seventh day must be taken to mean an increase in absorption in the later hours after feeding. That the level tends to drop quickly is shown again by the low figures at death. It is also seen that the organism did not distinguish between the two forms of aluminium administered. Both were absorbed. More is absorbed when the quantity in the food is large.

The aluminium found in the *liver* (see table 4A) is higher than for any previous group of animals excepting the fasting dogs, and the kidney is lowest for all groups. The amount in the *bile* is large, although not as large as in the preceding group. Again the *brain* and *spleen* hold appreciable amounts, but the difference between the values and those for the previous groups is not striking. Compared with control animals of the same group (dogs 50 and 51), this group shows more aluminium in the liver, bile and brain, and less in the kidney and spleen than the controls. Why the value for spleen in these two control dogs is so much higher than for any other group we cannot explain. Nor can we explain the very large aluminium content of the thyroid gland of these two dogs. There are no very remarkable differences in the other organs.

The quantity of aluminium in the *bile* is less than in the single feeding group, but this seems to be balanced by the increase in the liver. There

is less aluminium in the kidneys of this group than in any other, indicating that in this case at least the kidney is not so greatly concerned with the elimination of aluminium as the bile and liver.

It is interesting to note that the control animals maintained the same order of aluminium storage as in group II and group III, that is, liver, kidney and bile, while the experimental animals have established a new order of magnitude for storage,—kidney, liver and bile. This indicates again the use of the most efficient means of removing large quantities of aluminium. It must be mentioned again that when the animal body is subjected to large excesses of aluminium for a prolonged period, any one organ may quickly become saturated with this metal, so that even large quantities may exist in the blood, but the quantity in any particular organ may not increase.

This series of experiments leads us to the same conclusions we have reached before, that aluminium is absorbed from foods containing it, and appears in the blood and certain tissues, that the liver and bile are the organs chiefly concerned with its excretion. In addition we may conclude that the greatest absorption occurs after the fifteenth hour following the ingestion of food containing large quantities of aluminium.

The aluminium content of blood and tissues of dogs maintained on an aluminium-rich diet for four weeks. By prolonging the period of feeding of excessive quantities of aluminium one should be able to show whether the animal organism will establish a "tolerance" for the metal, whether some alteration in the method of dealing with it will occur, or whether the aluminium content of the blood and tissues can be pushed to still higher levels.

Table 5 shows the results obtained by the feeding of large quantities of aluminium for a period of one month. Here the changes are very striking. Considering first the average figures for the blood of dogs 52, 53 and 54, it is seen that the aluminium found in the blood the second, fourth and sixth hours after feeding diminishes steadily, week by week, so that by the fourth week there is found little more than one-third the amount found in the first week in the same time interval. The individuals showing this very strikingly are dogs 53 and 54. A similar thing happens in the case of the alum-phosphate animals (55, 56 and 57) except that the level tends to rise slightly after the third week. Dog 56 illustrates this especially well. On the whole, however, the tendency is distinctly toward a lowering of the quantity of aluminium in the blood.

This points to a decreased absorption. But absorption is still probably taking place as some of the figures obtained at the death of the animal show. It would seem that the tendency is for less and less aluminium to be absorbed as the weeks go on, but the process does not stop, it merely takes place more slowly. Possibly the permeability of the capillaries of

the intestinal wall has been altered by exposure to excessive quantities of aluminium compounds over a long period.

While there is some variation among individuals of the same group, those variations are not very striking, and the averages obtained are lower than for the dogs of groups 3 and 4.

The aluminium content of the blood of the two control dogs decreases also, but this can hardly be considered evidence of lowered aluminium absorption, but rather that, with a food relatively free of aluminium, such amounts of the metal as may have been stored in the body have tended to be eliminated. The aluminium in the blood of dogs 58 and 59 fell to the level for fasting dogs.

A decrease in the absorption of aluminium is evidenced by the slight decrease in the aluminium of the liver (see table 5A) and the marked decrease in the bile as compared with dogs of groups III and IV. Dogs 52, 53, 55 and 58 show more aluminium in the kidney than in the liver, while the others show more in the liver.

Considering all six aluminium fed animals together it is evident that although all the values are higher, with the exception of the kidney, than the control animals of this series the differences are less marked than in the other two groups (III and IV).

One might expect to find largely augmented quantities in some other organ to account for the smaller quantities found here. It has been said that the spleen and pancreas are especially rich in aluminium after the prolonged administration of food containing it (15). Apparently this is not correct for the spleen has little more than that of the control animals of this group, and about the same amount as those obtained from dogs fed aluminized food only once. Nor has the brain content increased, but has rather tended to decrease, when compared with that for the single feeding or one week dogs. The changes in the other organs do not seem remarkable.

In general, the evidence seems to point to a decreased absorption when a diet containing large amounts of aluminium is fed over a protracted period. The lessened quantity in the bile possibly indicates lessened excretion because of a lower blood level.

Aluminium content of blood and tissues of dogs maintained on an aluminium-rich diet for twelve weeks. This experiment was planned with the same factors in mind as in the preceding group. It was desired to determine by what mechanism the organism adapts itself to the long continued administration of aluminium.

Table 6 shows the results obtained for the blood after a twelve weeks period of such feeding. In general the results coincide with those for the preceding group. The aluminium in the blood tends to decrease as the weeks pass. There is some variation which is not dependent on the time when the blood samples were taken, or on the condition of the animals.

This experiment was done during the summer months. Usually the animals ate well the first three or four weeks, then the appetite failed, they might even refuse to eat for two or three days. Usually when appetite failed markedly milk was given for a few days in addition to the food. This generally resulted in improvement in appetite. Sometimes yeast was also given.

Dog 64, showing the lowest average for the whole period began to lose appetite very soon after the experiment began. A skin disease developed, the animal being covered with abscesses which became large bleeding sores. It became so sick it would not attempt to eat or even move. It was given milk, a quart at a time, by stomach tube, for a few days. Improvement started at once, by the end of the experiment it was eating all its food, the skin had healed and new hair was growing. Even under these various conditions the aluminium in the blood did not change much, maintaining a fairly constant low level after the third week except for the ninth week when it rose 100 per cent, then dropped again.

Dogs 63 and 65 maintained their appetites and good nutritional condition during the entire period of the experiment, appetite beginning to fail only in the last week.

That the quantity of aluminium in the blood is not dependent on the absolute amount ingested is again illustrated. Dog 60 ate well the first four weeks, the blood value varying during the time from a high figure to a low one. The seventh week, when the animal was not eating well, a high figure appears. In the later weeks, after the appetite had improved, the aluminium falls to a fairly low level.

By comparing the average blood content of this group with that of group V, it is found that the values are practically the same except for the alum phosphate dogs, for which the level is slightly lower. The averages for blood of dogs fed both types of biscuits are low in this experiment, but still slightly higher than for controls, indicating that even though absorption has decreased markedly it has not stopped altogether.

As in the preceding groups (see table 6A), liver and bile are chiefly concerned with excretion and storage. The average content of all the organs tends to be slightly higher for the twelve week animals than for the four week ones, except for controls, which are much the same in the two groups. While absorption has seemed to be decreased, storage in the organs has gone on, as well as efficient excretion in the bile.

The experiments of Myers and Morrison (16) in so far as they are comparable with our own show quantitative rather than qualitative differences.

General discussion. These experiments contain many interesting suggestions relative to the behavior of the animal body toward aluminium, and perhaps toward metallic ions in general.

We know the absurdity of crediting to the absorbing elements of the gastro-intestinal tract any occult discretionary power with respect to the

substances to which they are exposed. The body absorbs substances, regardless of their beneficient or harmful effects, in accordance with physico-chemical laws. The process goes on in a definite way, and depends on the relative concentrations and solubilities in the various components of the body. The tendency with respect to any given substance is for a shifting equilibrium to be established between the various organs, the blood, the lymph and the gastro-intestinal tract.

In these experiments such an equilibrium is definitely indicated. It is seen that the aluminium content of the blood shifts from higher to lower levels during the experiments, depending in a general way on the amount of aluminium ingested, with the exception of the last two experiments, when another factor, namely, permeability of the absorbing elements, has presumably altered.

It is apparent also that the equilibrium is changed with some difficulty since the ingestion of large amounts of aluminium-containing foods does not often result in a large increase in the blood or tissues. The general trend is toward an increase, but the establishment and maintenance of a high blood level by means of feeding seems impossible.

While aluminium was found in the blood of all but two of the animals tested we are unwilling to state that aluminium is a "physiological" metal in the sense that calcium or magnesium is. Since the amount varies considerably we are inclined toward the viewpoint that it should be classed rather with copper and arsenic.

If it should be proved a "physiological" metal, that is, a necessary and indispensable ion, it is difficult to define just what the function might be. Stoklasa (17) assigns to aluminium important functions in plant life, especially in photosynthesis, germination and growth. Balls (18) recalls the suggestion of Papillon that aluminium may act as a substitute for metals, notably iron, in which the diet is deficient. Balls himself, on the basis of rather scanty evidence, suggests that aluminium may replace iron in ferruginous proteins. Bertrand (19) names aluminium as one of the metals always occurring in the animal body, which may act as catalysers in vital processes. That it does not catalyse reactions occurring during muscular activity is suggested by its occasional absence from both heart and voluntary muscle. There have been suggestions that aluminium plays a rôle in reproduction (20) (21).

Table 7 summarizes the averages for the various groups. While caution is necessary in drawing conclusions from averages, this method is more fair, considering the large number of animals, than comparing individuals. It is to be expected that individuals may occasionally be found which show the contrary trend. In this discussion we confine ourselves entirely to these larger, more obvious and general tendencies.

Considering the organs first in respect to the aluminium *per unit* of *tissue* we are surprised at the very high result for the thyroid, which

contains nearly three times as much as the next highest tissue. But the thyroid, as has been pointed out before, because of its very small size, cannot be considered an important storage depot for aluminium. But the fact that this tissue picks out aluminium in such large amounts would seem to have some significance. Is aluminium necessary to the metabolism of the thyroid? Is aluminium a vital constituent of the gland or a fortuitous impurity attached to colloids? That this is not a property of glands in general is shown by the relatively small amount in the other glands examined, namely, adrenal, ovary and testicle. No aluminium was found in any of the ovaries examined. It is sometimes present, sometimes absent in the testicle and adrenal. In the adrenal the amount present has no discernible relation to the experimental procedure.

Still considered on the basis of unit content, the spleen is the second richest organ, containing over 50 per cent more aluminium than the brain, which in turn contains nearly 90 per cent more than the liver and kidney.

The bile is, in every case, very rich in aluminium. This shows conclusively that here is the most important channel for excretion. It is probable that some of this excreted aluminium is again absorbed.

When the aluminium stores of the organs are considered in order of actual importance because of size, the liver must be given first place. The brain is next in importance with the kidney and spleen nearly equal, for while the spleen weighs less than the kidney its aluminium content is greater. The heart and voluntary muscle are much less important as storage depots for aluminium.

The general differences noticed between the animals fed once, or for one, four or twelve weeks merely illustrate different stages in the same process, namely the physiological adjustment to the continued administration of large amounts of aluminium in the food. At first, after a single feeding, the body adjusts by absorbing relatively large quantities, storing a large share in the brain, spleen and other tissues, and excreting much through the bile. At the end of one week's feeding the body is still adjusting in much the same way and to about the same extent. But after a month's exposure to this treatment absorption is definitely decreasing and storage and excretion concomitantly, though excretion through the bile is still large. After three months, absorption has decreased further, but the aluminium absorbed is treated in the same manner as before.

We are not in a position to state whether decreased absorption means decreased permeability, that is, injury, of the capillaries of the intestinal tract. This may very well be another purely normal physiological adjustment. On the other hand, can lessened absorption be regarded as a protective mechanism against possible injury by aluminium? Its behavior in this respect is strikingly like that of arsenic. There was no

evidence, from observation of our animals, of any impairment in health due to these dietaries.

CONCLUSIONS

1. Aluminium occurs in the blood and tissues of normal fasting dogs.

2. Aluminium occurs in slightly larger amounts in the blood and tissues of dogs fed a diet to which no aluminium had been added. Therefore aluminium is present in and absorbed from the ordinary diet.

3. Aluminium is promptly absorbed in small quantities following a single feeding of food to which it has been added.

4. Aluminium continues to be absorbed when aluminium-rich diets are fed for various periods of time.

5. After prolonged periods of feeding with food rich in aluminium, absorption of the metal decreases.

6. Concomitant with the decrease in absorption there is a decrease in storage and excretion.

7. The aluminium absorbed circulates in the blood, is stored especially in the liver, brain, kidney, spleen and thyroid.

8. The bile is the chief excretory path for aluminium.

BIBLIOGRAPHY

(1) Smith, E. E. Aluminium compounds in food. (Hoeber) 1928, New York.
(2) Myers, C. N. and C. Voegtlin. Pub. Health Reports, 1914, xxix, 1625.
(3) Kastyal, L. Magyar Orvasi Archivum, 1926, xxvii, 237. From C. A.
(4) Archangelskii, V. M. Arch. Biol. Sciences, 1924, xxiv, 163.
(5) Abrahamson, E. M. and E. G. Miller, Jr. Proc. Soc. Exper. Biol. Med., 1925, xxii, 438.
(6) Britton, H. T. S. Journ. Chem. Soc., 1925, cxxvii, 2110.
(7) Blum, W. Journ. Amer. Chem. Soc., 1916, xxxviii, 1282.
(8) Greenfield, R. E. and A. M. Buswell. Ibid., 1922, xliv, 1435.
(9) Connor, S. D. Journ. Amer. Soc. Agron., 1921, xiii, 113.
(10) Underhill, F. P. and F. I. Peterman. This Journal, 1929, xc, 1.
(11) McCollum, E. V., O. S. Rask and F. E. Becker. Journ. Biol. Chem., 1928, lxxvii, 753.
(12) Langworthy, C. F. and P. T. Austin. The occurrence of aluminium in vegetable products, animal products and natural waters. New York, Wiley, 1904.
(13) Gies, W. J. Journ. Amer. Med. Assoc., 1911, lvii, 816.
(14) Hammarsten, O. and S. G. Hedin. Physiological chemistry. Transl. by J. A. Mandel, 7th ed., Wiley, 1915, p. 414.
(15) Kahn, M. Biochem. Journ., 1912, ii, 235.
(16) Myers, V. C. and D. B. Morrison. Journ. Biol. Chem., 1928, lxxviii, 615.
(17) Stoklasa, J. Über die Verbreitung des Aluminiums in der Natur. Fischer, Jena, 1922.
(18) Balls, A. K. Dissertation. Columbia, 1920.
(19) Bertrand, G. Bull. Soc. Sci. Hyg. Alimentaire, 1920, viii, 49.
(20) Daniels, A. L. and M. K. Hutton. Journ. Biol. Chem., 1925, lxiii, 143.
(21) Mitchell, H. S. and L. Schmidt. Journ. Biol. Chem., 1926, lxx, 471.

STUDIES IN THE METABOLISM OF ALUMINIUM

III. Absorption and Excretion of Aluminium in Normal Man

FRANK P. UNDERHILL and F. I. PETERMAN

From the Department of Pharmacology and Toxicology, Yale University, New Haven, Conn.

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Aluminium compounds form a large part of the natural environment of plant and animal life. Escape from dust containing it is impossible. Significant amounts are found in many foods. In view of the fact that no substance can be considered absolutely insoluble in the laboratory of the animal body, it is to be expected that aluminium will find its way into the body in accordance with physico-chemical laws. An equilibrium must be established between the aluminium on what may be termed the outside of the body membrane, that is, the intestinal tract and the lung, and that on the inside—the tissues themselves. Considering therefore the aluminium which in one form or another must find its way into the body tube in food and into the respiratory tract in dust, it would be surprising indeed if it were not present in some parts of the body.

The results reported for the determination of aluminium in the blood of man are unconvincing (1). Our work (2) shows that aluminium is nearly always found in the blood of dogs, the amount increasing slightly after the feeding of aluminium-rich food. It seems probable that the negative results reported for man have been due to the shortcomings of the analytical methods used. The delicacy and reliability of our own method, outlined in the first paper of this series of studies in the metabolism of aluminium, prompted us to re-investigate the question of absorption of aluminium in man.

In this study it was desired to determine the following points:

1. Is aluminium present in the blood in normal man?
2. Is aluminium absorbed from food containing it?
3. Is aluminium excreted in the urine?

Two experiments were performed. In the first fifteen, in the second twelve subjects were used. These subjects were healthy young people, students in the Yale School of Medicine. In the first experiment two subjects, nos. 12 and 13, and in the second experiment, subject 7, were young women. To all we extend thanks for their valuable coöperation in this study.

METHODS. *The determination of aluminium in human blood.* It is desirable, in working with human beings to make all analyses with the smallest possible amount of blood. In order to adapt the method previously reported (3) to the determination of aluminium in human blood the following procedure was used. Ten cubic centimeters of blood were drawn from an arm vein. Two 5 cc. portions were measured into silica crucibles, dried, ashed and dissolved as usual. Five cubic centimeters of the ash solution was shaken out with ether and ammonium thiocyanate. Then 5 cc. of the iron-free solution were transferred to a 10 cc. volumetric flask, and 2.5 cc. of the glycerine-citric acid mixture, and 1 drop of 0.25 per cent mono-sodium alizarine sulphonate added. The pH was adjusted as previously described. Then 0.5 cc. of 0.25 per cent alizarine solution and 0.2 cc. concentrated ammonium hydroxide were added, the neck of the flask washed, and the volume made to about 9 cc. Flasks stood, stoppered, for 24 hours, when 0.5 cc. of glacial acetic acid was added, the volume made to 10 cc., mixed and compared at once in the colorimeter. Standards and blanks were prepared simultaneously. Standards contained 0.00025, 0.0005, 0.00075, 0.001, 0.002 mgm. aluminium.

This manipulation must be executed with the utmost care as colors are very delicate, and color differences small. Any quantity of aluminium found in the blanks was substracted from that in the unknown. All determinations were done in duplicate.

Determination of aluminium in urine. In the first experiment 10 cc. portions, in the second 25 cc. portions of urine were dried, ashed and dissolved. Usually the determination was made directly on 5 cc. of the ash solution without previously shaking out with ammonium thiocyanate and ether. When the urine was found to contain enough iron to interfere with the color iron was removed as usual.

Preparation of the aluminium containing biscuit. The aluminium containing biscuit used in these experiments was prepared with a well known brand of "alum-phosphate" baking powder, the composition of which is given in a previous paper (2). In the first experiment the biscuits were made according to the directions given on the can, and contained the following:

	grams
Flour	946
Baking powder	30
Salt	10
Lard	40
Water q.s.	

The biscuits in the second experiment were made according to a recipe in a widely used cook book (4) and contained:

	grams
Flour	4034
Baking powder	445
Salt	95
Lard	1000
Water q.s.	

When griddle cakes were made, the dry mixture used was the same, but more fluid was added.

The manipulation was carried out as is usual in household cooking.

The first experiment. Fifteen subjects reported at the laboratory at 9 a.m. after fasting since 7 p.m. of the previous evening. A blood sample, 10 cc., was drawn from the median basilic vein. A urine sample was collected in a paraffin coated bottle. These samples were considered normal with respect to contained aluminium. Ten subjects were given aluminium biscuit, five yeast bread.

The meal was given after all the samples had been collected. The meal consisted of bread or biscuit, butter, boiled ham, distilled water.

Each subject ate as much as he desired, the weight of bread or biscuit being recorded. Time was counted from the minute the meal was finished. The second blood samples were taken from each of three subjects at the end of the first, second, fourth, sixth and eighth hour. Urine samples were collected from all at the end of the second, fourth, sixth and eighth hour. No food was allowed during this period, but distilled water was allowed *ad libitum*.

The second experiment. Twelve subjects reported at the laboratory at 9 p.m., having fasted since noon. A blood and urine sample was collected from each. At 10 p.m. a meal consisting of roast beef, gravy, biscuit or bread, and butter was served. Each subject ate as much as he wished, weights of biscuit or bread being recorded.

The subjects reported at the laboratory at 8 a.m. the following morning, having collected the night urine in paraffined bottles. Breakfast, consisting of griddle cakes, sugar syrup, butter and coffee was served. Controls ate yeast bread or toast. Urine samples were collected during the day at 10 a.m., 2, 6 and 10 p.m. Two blood samples were taken from each subject at intervals of eight hours as is shown in table 3. Time was counted from the time of the meal the previous evening.

Lunch consisted of alum biscuit, or yeast bread, butter, boiled ham and coffee. Supper consisted of cold roast beef, boiled ham, alum biscuit or yeast bread, and butter. No other food was eaten during the experimental period. Cream and sugar were allowed in coffee. The coffee was made with distilled water and found to contain only a trace of aluminium. Distilled water was allowed *ad libitum*.

RESULTS. *The blood.* The tables show the results of these experiments. It will be seen that aluminium often occurs in the blood of normal man. The amount present is small, the highest value found being 0.210 mgm. per 100 cc. (subject 1, first experiment). The amount is usually much less than this, the average being 0.060 mgm. for the first and 0.059 mgm. for the second experiment. When no value is given for the aluminium of the blood it means that the amount present is less than 0.001 mgm. per 100 cc. In the first experiment three subjects showed no detectable aluminium in the blood; in the second, two. Traces, that is amounts too small to estimate accurately, were shown by two in the first, by four in the second.

But comparison of tables 1 and 3 shows that the aluminium content of the normal blood of the same person may vary from a non-detectable to an appreciable amount. Below the results for the two experiments are tabulated.

SUBJECT	FIRST EXPERIMENT	SECOND EXPERIMENT
LaV	0.124	Trace
E. T.	Trace	0.108
K.	0.000	0.104
P.	0.124	Trace
W.	0.096	0.000
R.	Trace	0.110
Subjects showing less change are:		
B.	0.210	0.167
J.	0.102	0.164
C.	0.016	Trace
G.	0.000	0.056
H.	0.053	0.000
R.T.	0.026	Trace

In the first experiment after the ingestion of aluminium-rich food the aluminium content of the blood was found to be definitely increased in two subjects, slightly in two others. In the first two the increase was over 100 per cent in each case. This increase has no apparent relation to the amount of aluminium-containing food ingested or the time after the meal.

In the second experiment definite increases in the blood are found in only two cases, the increase appearing 12 to 14 hours after the ingestion of large amounts of aluminium-containing food. One of these subjects (LaV.) did not show an increase in the first experiment. In all the others, including control subjects, the tendency was for any aluminium in the blood to decrease during the course of the experiment.

TABLE 1

The aluminium content of blood and urine after a single ingestion of aluminium containing food

HOUR	Al/100 cc. BLOOD	URINE VOLUME	Al/100 cc. URINE	TOTAL Al URINE	BISCUIT	Al/100 cc. BLOOD	URINE VOLUME	Al/100 cc. URINE	TOTAL Al URINE	BISCUIT	Al/100 cc. BLOOD	URINE VOLUME	Al/100 cc. URINE	TOTAL Al URINE	BREAD (YEAST)
	Subject 1 (B. wt. 160 pounds)					Subject 2 (LaV. wt. 155 pounds)					Subject 8 Control (E. T. wt. 146 pounds)				
		cc.		*mgm.*	*grams*		*cc.*		*mgm.*	*grams*		*cc.*		*mgm.*	*grams*
0	0.210	25	0.075	0.019	179.5	0.124	7.5	0.000	0.000	340	Trace	34	0.000	0.000	181
1	0.200					0.102					Trace				
2		37	0.000	0.000			47	0.000	0.000			65	0.000	0.000	
4		100	0.065	0.065			74	0.000	0.000			97	0.000	0.000	
6		67	0.072	0.048			170	0.000	0.000			170	0.000	0.000	
8		42	0.074	0.031			113	Trace	Trace			65	0.000	0.000	
				0.163					Trace					0.000	
	Subject 4 (K. wt. 155 pounds)					Subject 5 (W. wt. 145 pounds)					Subject 3 Control (J. wt. 138 pounds)				
0	0.000	37	0.098	0.036	213	0.096	55	0.050	0.025	141	0.102	15	0.103	0.015	170
2	0.000	41	0.000	0.000		0.204	59	0.049	0.029		0.094	24	0.096	0.023	
4		64	0.000	0.000			36	0.088	0.032			83	0.100	0.083	
6		62	0.102	0.063			63	0.080	0.050			87	0.051	0.045	
8		98	0.099	0.098			65	0.051	0.033			61	0.052	0.031	
				0.197					0.169					0.197	
	Subject 7 (C. wt. 135 pounds)					Subject 9 (R. T. wt. 147 pounds)					Subject 6 Control (H. wt. 190 pounds)				
0	0.016	16	0.000	0.000	235	0.026	148	0.078	0.038	137	0.053	19	0.053	0.010	227
2		45	Trace	Trace			190	0.078	0.148			47	0.053	0.025	
4	0.008	55	0.000	0.000		0.056	167	Trace	Trace		Trace	69	0.001	0.001	
6		60	0.098	0.059			108	Trace	0.000			77	0.000	0.000	
8		53	0.096	0.051			67	0.000	0.000			67	0.000	0.000	
				0.110					0.186					0.036	

	Subject 10 (N. wt. 215 pounds)					Subject 11 (G. wt. 138 pounds)					Subject 15 Control (R. wt. 137 pounds)				
0	0.032	170	0.000	0.000	255	0.000	67	0.000	0.000	205	Trace	25	0.024	0.006	152
2		100	0.000	0.000			48	0.088	0.043			70	0.016	0.011	
4		145	0.053	0.077			50	0.041	0.021			155	0.016	0.024	
6	0.026	160	0.000	0.000		Trace	156	0.049	0.077		0.000	135	0.016	0.022	
8		105	0.000	0.000			139	0.030	0.042			150	0.040	0.060	
				0.077					0.183					0.123	
	Subject 13 (P. wt. 110 pounds)					Subject 14 (M. wt. 150 pounds)					Subject 12 Control (L. wt. 119 pounds)				
0	0.124	158	0.021	0.030	166	0.120	187	0.050	0.094	128	0.000	185	0.048	0.089	
2		86	0.020	0.018			51	0.060	0.030			34	0.000	0.000	
4		150	0.020	0.030			52	0.053	0.028			102	0.000	0.000	
6		70	0.026	0.018			39	0.060	0.023			50	0.024	0.012	
8	0.095	42	0.019	0.008		0.140	24	0.047	0.011		0.000	45	0.032	0.011	
				0.104					0.186					0.112	

There is here some evidence that greatest absorption of aluminium occurs in the later hours after the ingestion of food to which aluminium has been added. Dogs (2) also show this delay. Does this mean that aluminium is not so easily absorbed from the upper part of the intestinal tract as from a lower part?

Where an increase of aluminium in the blood occurs, after the ingestion of aluminium-containing foods, we may be sure that this will be excreted later, in part at least, by the kidney.

The urine. The urine of normal subjects often contains aluminium. In the first experiment the normal urine of ten subjects showed aluminium,

TABLE 2

Summary—Averages—First experiment

TIME	BLOOD Al/100 cc.		URINE Al/100 cc.	
	Experimental subjects	Controls	Experimental subjects	Controls
Before meal	0.075	0.031	0.037	0.046
After meal	0.082	0.019		
2 hours			0.028	0.033
4 hours			0.033	0.023
6 hours			0.047	0.018
8 hours			0.042	0.025

	mgm.
Average for blood of all subjects before meal	0.060
Average for blood of all subjects after meal	0.062
Average for urine of all subjects, before meal	0.040
Average for total aluminium in urine, experimental subjects	0.137
Average for total aluminium in urine, control subjects	0.096

the average for all being 0.040 mgm. per 100 cc., a value two-thirds that of the blood value. After the ingestion of an aluminium-rich meal, the urine may be aluminium-free for a number of hours then aluminium may appear again, illustrated by subjects 1, 4, 7, 10 and 15. Or the rate of excretion of aluminium, as judged by the amount per 100 cc. for 2-hour periods may change very little (subjects 13 and 14), or the rate of excretion may steadily decrease (subject 9), or an initial rise may appear, followed by a progressive decrease (subject 11). The aluminium content of the urine of one control subject rose slightly (subject 15) but not above the preliminary level.

The difference in the amount of aluminium excreted by the experimental and control subjects in the first experiment is not great, as is shown in

table 2. The average total excretion for those ingesting aluminium is slightly higher than for the others.

In the second experiment the average aluminium content of normal urine was somewhat less than in the first, two subjects showing no aluminium, another only a trace.

The rate of excretion of aluminium was found to increase at some interval of the experimental period in subjects 1, 2, 4, 5, 7, 11 and 12, the latter a control subject. In subject 8, who showed a decided increase in the blood content, the amount of aluminium per 100 cc. of urine decreased during the experiment. Subject 10, after aluminium had been absent for a number of hours, showed a small amount. In subject 12 (control), the gain in the urine is partly accounted for by the loss from the blood. Subject 3 (control) showed no decrease in the amount of aluminium in the blood during the experimental period, and no aluminium appeared in the urine.

The total aluminium excretion for 24 hours was in no case over 0.5 mgm. and in one subject no aluminium, in another only a trace was excreted during the entire period. The total excretion of aluminium is decidedly higher for those who ingested aluminium-rich food than for corresponding controls.

From results for the urine we are led to believe that even though aluminium is often not detected in the blood it must at times be present since the urine shows definite amounts of aluminium. That the urine often shows a definite increase after the ingestion of aluminized foods points to absorption even though the increase in the blood is not always detected. While we know the condition of the blood only at the moment the sample is taken it is unlikely that large fluctuations will occur in the intervals.

The differences between the results for man and dog may be attributed to the differences in physico-chemical balance in the two different organisms, the human being a much more sensitively adjusted mechanism than the dog. It is to be inferred that the tendency of the body to keep a delicate and constant balance among the various inorganic ions cannot be easily disturbed by the ingestion of relatively large amounts of any one ion. If one may draw an analogy from experiments with the dog, one method of maintenance of this balance is by elimination of aluminium through the intestine (see paper IV).

TABLE 3

The aluminium content of blood and urine after the ingestion of aluminium containing foods

TIME	HOUR	Al/100 cc. BLOOD	URINE VOLUME	Al/100 cc. URINE	TOTAL Al URINE	BISCUIT	Al/100 cc. BLOOD	URINE VOLUME	Al/100 cc. URINE	TOTAL Al URINE	BISCUIT	Al/100 cc. BLOOD	URINE VOLUME	Al/100 cc. URINE	TOTAL Al URINE	BREAD (YEAST)
		Subject 1 (K. wt. 155 pounds)					Subject 2 (W. wt. 145 pounds)					Subject 3 Control (R. wt. 137 pounds)				
			cc.		*mgm.*	*grams*		*cc.*		*mgm.*	*grams*		*cc.*		*mgm.*	*grams*
Sat. 10 p.m.	0	0.104	240	0.020	0.028	162	0.000	143	0.011	0.016	62	0.110	80	0.000		70
Sun. 8 a.m.	10	0.105	210	0.069	0.153	260	0.000	195	0.021	0.051	260	0.111	135	0.000		106
10 a.m.	12		58	0.059	0.034			50	0.051	0.025			90	0.000		
12 m.	14					100					114		150	0.000		75
2 p.m.	16		190	0.044	0.084			140	0.042	0.058			50	0.000		
4 p.m.	18	0.096					0.000					0.111				
6 p.m.	20		220	0.059	0.130	65		100	0.042	0.042	42		375	0.000		62
8 p.m.	22												140	0.000		
10 p.m.	24		115	0.048	0.055			90	0.042	0.038			125	0.000		
					0.484	587				0.230	478			0.000		313
		Subject 4 (C. wt. 135 pounds)					Subject 5 (R. T. wt. 147 pounds)					Subject 6 Control (H. wt. 195 pounds)				
Sat. 10 p.m.	0	Trace	100	0.000	0.000	134	Trace	50	0.026	0.013	127	0.000	187	0.014	0.027	165
Sun. 8 a.m.	10		137	0.019	0.026	130		163	0.041	0.067	260		239	0.000	0.000	205
10 a.m.	12	Trace	58	0.019	0.011		Trace	70	0.032	0.022		0.000	66	0.000	0.000	
12 m.	14					150					96					113
2 p.m.	16		112	0.000	0.000			378	0.025	0.094			173	0.000	0.000	
4 p.m.	18															
6 p.m.	20	Trace	160	0.000	0.000	97	Trace	287	0.029	0.084	69	0.000	177	0.020	0.035	110
8 p.m.	22															
10 p.m.	24		95	0.000	0.000			113	0.025	0.028			131	0.000	0.000	
					0.037	511				0.308	552				0.062	593

		Subject 7 (P. wt. 110 pounds)					Subject 8 (G. wt. 138 pounds)					Subject 9 Control (E. T. wt. 146 pounds)				
Sat. 10 p.m.	0	Trace	39	0.019	0.007	119	0.056	338	0.029	0.099	176	0.108	95	Trace		116
Sun. 8 a.m.	10		265	0.030	0.079	152		75	0.030	0.022	400		199	Trace		108
10 a.m.	12		200	0.010	0.020			60	0.029	0.017			70	Trace		
12 m.	14	Trace				102	0.056				73	0.106				116
2 p.m.	16		652	0.024	0.152			175	0.009	0.016			257	Trace		
4 p.m.	18															
6 p.m.	20		572	0.029	0.168	71		223	0.010	0.022	106		210	Trace		70
8 p.m.	22	Trace					0.153					0.050				
10 p.m.	24		447	0.009	0.040			118	0.011	0.013			130	Trace		
					0.466	444				0.189	755				Trace	410
		Subject 10 (LaV. wt. 155 pounds)					Subject 11 (B. wt. 160 pounds)					Subject 12 Control (J. wt. 138 pounds)				
Sat. 10 p.m.	0	Trace	120	0.029	0.035	186	0.167	58	0.019	0.010	120	0.164	92	0.019	0.018	240
Sun. 8 a.m.	10		750	0.000	0.000	340		160	0.078	0.125	260		240	0.000	0.000	95
10 p.m.	12		41	0.000	0.000			60	0.009	0.006			61	0.020	0.012	
12 m.	14					183					143					108
2 p.m.	16	Trace	102	0.000	0.000		0.111	235	Trace	Trace		0.106	146	0.058	0.085	
4 p.m.	18															
6 p.m.	20		220	0.019	0.042	86		210	Trace	Trace	90		174	0.057	0.100	67
8 p.m.	22															
10 p.m.	24	0.086	165	0.020	0.032		0.108	165	0.020	0.033		0.109	102	0.057	0.058	
					0.109	795				0.174	613				0.273	510

TABLE 4

Summary—Averages—Second experiment

TIME	BLOOD Al/100 cc.		URINE Al/100 cc	
	Experimental subjects	Controls	Experimental subjects	Controls
Normal	0.041	0.095	0.019	0.008
First blood sample	0.034	0.081		
Second blood sample	0.055	0.068		
10 hours			0.036	Trace
12 hours			0.026	0.005
16 hours			0.018	0.014
20 hours			0.025	0.019
24 hours			0.021	0.014

	mgm.
Average, blood of all subjects before experiment	0.059
Average, urine of all subjects before experiment	0.016
Average, total urine excretion, experimental subjects	0.250
Average, total urine excretion, control subjects	0.084
Average, 24 hour excretion, experimental subjects	0.024
Average, 24 hour excretion, control subjects	0.073

CONCLUSIONS

1. Aluminium may or may not occur in the blood of normal man. If it does occur the amount is not large, 0.21 mgm. per 100 cc. being the maximum found.

2. The aluminium content of the blood of the same normal person may vary from time to time between narrow limits.

3. The aluminium content of the blood may show definite increase after ingestion of aluminium-rich food. More often an increase is not detected.

4. The control subjects fed on food presumably poor in aluminium never showed an increased content of aluminium in the blood. On the contrary, the blood aluminium content usually decreased under these conditions.

5. The urine of normal man may or may not contain small amounts of aluminium.

6. The aluminium in the urine tends to increase after the ingestion of aluminium-rich foods.

7. Even after the ingestion of large amounts of aluminium-rich food the total urinary excretion for 24 hours does not exceed 0.5 mgm. aluminium.

BIBLIOGRAPHY

(1) SMITH, E. E. Aluminium compounds in food. Hoeber, 1928, New York.
(2) UNDERHILL, F. P. AND F. I. PETERMAN. This Journal, 1929, xc, 15.
(3) UNDERHILL, F. P. AND F. I. PETERMAN. This Journal, 1929, xc, 1.
(4) FARMER, F. Boston cooking school cook book, p. 70. New ed., revised and enlarged. Little Brown Co., 1925.

STUDIES IN THE METABOLISM OF ALUMINIUM

IV. The Fate of Intravenously Injected Aluminium

FRANK P. UNDERHILL, F. I. PETERMAN AND S. L. STEEL

From the Department of Pharmacology and Toxicology, Yale University, New Haven, Conn.

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The fate of aluminium after intravenous injection has not been extensively studied. Steel (1), in four dogs, found 5.27 to 11.11 per cent of intravenously injected aluminium in the feces in the three days following the injection. The remainder is not accounted for.

Recently Myers and Morrison (2) have shown that when aluminium is parenterally introduced into dogs it is widely distributed in the tissues and is only slowly excreted.

Aluminium is very gradually absorbed by the dog from aluminium-rich food, the quantity in the blood seldom rising to a high figure (3). Thus far one has been unable to cause a large and sudden increase in the blood value by means of feeding. In the present study an attempt has been made to learn in what ways the body would react to a sudden increase in the aluminium content of the blood. An amount of aluminium was injected which was slightly greater than the largest amount found in the blood after feeding aluminium-rich food. It was planned to follow the rate of disappearance from the blood and the time of appearance in other body fluids, as well as the rate of increase and decrease.

Procedure. Dogs under morphine-atropine-ether, or amytal anesthesia were used. The femoral vein was exposed for injection purposes. It was not ligatured, injection being made slowly through a hypodermic needle from a burette. The femoral artery on the opposite side was exposed for blood collection. This was usually clamped off, but a free flow of a few cubic centimeters of blood was allowed before the sample was taken. Blood samples were collected directly into Ostwald pipettes, no oxalate being used.

Urine was collected by means of a catheter. Lymph was obtained from the thoracic duct. Bile was collected from the common bile duct after the gall bladder had been tied off.

Both lymph and bile were collected directly into weighed silica crucibles and samples weighed. When bile was being collected, 15 to 20 cc. of 0.4

per cent hydrochloric acid was injected into the duodenum to stimulate the flow. This was not usually repeated.

Stomach and intestinal loops were made by the insertion of large cannulae. The entire stomach and large intestine were washed, and a loop of small intestine about three feet in length, below the entrance of the bile duct, was washed with warm saline, the last washing before the injection being used as a control sample. In only one case did any of the washings contain more than a small trace of blood.

Every precaution was taken to prevent contamination of samples.

The solutions used for injection were aluminium sulphate or aluminium chloride dissolved in normal saline. In one case 10 mgm. of aluminium per kilo were given but the dog died suddenly, and the aluminium of the blood was too high to be accurately estimated by the method used. The usual amount given was 1 or 2 mgm. of aluminium per kilo. The injection was made slowly. The animal was kept warm.

ANALYTICAL METHODS. Aluminium was determined by means of the method already described (4).

Phosphate was determined by a modification of the Bell-Doisy (5) method as follows: 50 cc. of the saline washings were evaporated to dryness in a silica crucible and ashed at a low red heat. The ash was dissolved in 5 cc. of 2 per cent trichloracetic acid and 1 cc. of sodium sulphite, 2 cc. molybdate solution and 1 cc. of hydroquinone were added. Standards prepared at the same time contained 1 to 5 cc. of standard phosphate solution in a final volume of 9 cc. All phosphorus was thus determined as inorganic phosphate.

RESULTS. The protocols reproduced below illustrate the procedure and show the results obtained.

I. The examination of blood, urine and bile.

Experiment A

Dog, weight 14.2 kgm. Intravenous injection of $AlCl_3$ solution. Given 2 mgm. Al per kilogram = 71.0 cc. solution = 28.4 mgm. Al.

TIME	BLOOD, Al/100 cc.	BILE, Al/100 GRAMS	URINE, Al/100 cc.	REMARKS
	mgm.	*mgm.*	*mgm.*	
12:20	0.18		Trace	Normal. 15 cc. 0.4 per cent HCl injected into duodenum
12:35		None		Normal
12:40				Injection begun
1:00		0.72		
1:08				Injection finished
1:10	4.32			All urine pressed from bladder
1:24		0.83		
1:38	4.16			½ hour
2:00		0.70		

Experiment A—concluded

TIME	BLOOD, Al/100 cc.	BILE, Al/100 GRAMS	URINE Al/100 cc.	REMARKS
	mgm.	*mgm.*	*mgm.*	
2:08	4.01			1 hour
2:40			2.58	Volume 32 cc. Total excretion 0.83 mgm.
2:50		1.16		
3:08	3.54			2 hours
3:35		0.79		
4:18		0.70		
4:30			1.29	Volume 35 cc. Total excretion 0.45 mgm.
5:08	3.37	0.74		4 hours
5:55		0.66		
6:08	3.19			5 hours
6:55		0.62	1.57	Volume 48.5 cc. Total excretion 0.76 mgm.
7:08	2.69	1.06		6 hours

Total urine volume = 115.5 cc.
Total aluminium in urine = 2.04 mgm.
Total bile = 14.978 grams
Total aluminium in bile = 0.110 mgm.

Experiment B

Dog, weight 16.3 kgm. Intravenous injection $AlCl_3$ solution, 5 cc. = 2 mgm. Al. Given 1 mgm. Al per kilogram.

TIME	BLOOD, Al/100 cc.	BILE, Al/100 GRAMS	URINE, Al/100 cc.	REMARKS
	mgm.	*mgm.*	*mgm.*	
9:45			None	Normal
1:35	0.21		None	Normal; 20 cc. 0.4 per cent HCl injected into duodenum
2:30		0.196		Normal
2:32				Injection begun
2:57				Injection finished
3:27	2.51			½ hour
3:57	2.38			1 hour
4:22			2.60	Volume 10.2 cc. Excretion 0.27 mgm.
4:27	2.64			1½ hours
4:57	2.68	0.372		2 hours
5:27	2.60			2½ hours
5:57	2.72			3 hours
6:30			1.95	Volume 11.9 cc. Excretion 0.21 mgm. Al
6:57	2.79			4 hours
7:57	2.82			5 hours; 5 mgm./kgm. morphine subcutaneously
8:57	2.03			6 hours
9:57	1.06	1.010		7 hours

Total urine excretion = 22.1 cc.
Total aluminium in urine = 0.48 mgm.
Aluminium in liver = 0.85 mgm. per 100 grams
Total bile = 7.054 grams
Total aluminium in bile = 0.31 mgm.

II. The examination of blood, urine and lymph. In the first three of the following experiments aluminium solutions were injected, in the last the aluminium was administered per os in baking powder biscuits. In the first experiment an excessive dose of aluminium chloride was given and the dog died suddenly before the full amount had been injected. The protocol is included however as illustrating the excretion of aluminium through the lymph.

Experiment C

Dog, weight 13.6 kgm. Intravenous injection of $AlCl_3$ solution, 5 cc. = 10 mgm. Al. Given 10 mgm. per kilogram.

TIME	BLOOD, Al/100 cc.	LYMPH, Al/100 GRAMS	URINE, Al/100 cc.	REMARKS
	mgm.	*mgm.*	*mgm.*	
11:30		None		Normal
12:00	0.04	None	Trace	Normal
12:35				Injection begun; 1 cc. per minute
1:10	5.81	2.16	0.067	Died suddenly

Experiment D

Dog, weight 11.1 kgm. Intravenous injection of $AlCl_3$, 5 cc. = 2.0 mgm. Given 1 mgm. per kilogram. Amytal anesthesia.

TIME	BLOOD, Al/100 cc.	LYMPH, Al/100 GRAMS	URINE, Al/100 cc.	REMARKS
	mgm.	*mgm.*	*mgm.*	
2:30	0.21	Trace	None	Normal
2:40				Injection begun
3:00				Injection finished
3:15		0.48		
3:30	1.87			½ hour
3:35		0.45		
4:00	1.82			1 hour
4:04		0.25		
4:30	1.82			1½ hours
4:44		0.33		
5:00	2.22			2 hours
5:15		0.28		
6:00	2.05			3 hours
6:15		0.21		
6:20			4.82	Volume 14.7 cc. Excretion 0.71 mgm. Al
7:00	2.05			4 hours
8:00	2.13	0.28		5 hours
9:00	2.06	0.39		6 hours
9:10			2.32	Volume 8.0 cc. Excretion 0.19 mgm.
9:30	1.87			6½ hours

Total urine excretion = 22.7 cc.
Total aluminium in urine = 0.89 mgm.
Total lymph = 56.68 grams
Total aluminium in lymph = 0.160 mgm.
Aluminium content of liver = 0.68 mgm. per 100 grams

Experiment E

Dog, weight 15.6 kgm. Intravenous injection of $AlCl_3$ solution, 5 cc. = 1 mgm. Al. Given 1 mgm. per kilogram.

TIME	BLOOD, Al/100 cc.	LYMPH, Al/100 GRAMS	URINE, Al/100 cc.	REMARKS
	mgm.	*mgm.*	*mgm.*	
3:15		None	No urine	Normal
4:00	None	None	excreted	Normal
4:01				Injection begun
5:00		0.26		
5:05				Injection finished
5:30		0.35		
5:35	1.13			30 minutes
6:00		0.61		
6:05	1.15			1 hour
6:30		0.60		
7:05	1.11			2 hours
7:30		0.58		
8:05	1.11			3 hours

Aluminium content of liver = 0.58 mgm. per 100 grams
Total lymph = 46.458 grams
Total aluminium in lymph = 0.056 mgm.

Experiment F

Dog, weight 9.6 kgm. Fed meat and alum-phosphate biscuit. Amytal anesthesia.

TIME	BLOOD, Al/100 cc.	LYMPH, Al/100 GRAMS	REMARKS
	mgm.	*mgm.*	
6:50 a.m.			Fed
7:22			Amytal
10:25			Lymph collecting begun
10:55		0.114	
11:00	0.000		
11:25		0.087	
11:40	0.000		Lymph cannula cleaned
11:55			
12:30		0.079	
1:30	0.000	0.061	
2:30	0.000	0.060	
3:30	0.000	0.064	
4:30	0.000	0.101	

Total lymph = 45.78 grams
Total aluminium in lymph = 0.035 mgm.
Total urine excretion = 14.50 cc.

III. Excretion through the walls of the intestinal tract. The following experiments illustrate the excretion of aluminium through the walls of the intestinal tract. In the first experiment aluminium only was determined. In the others phosphate was also determined. In the last experiment the injection fluid consisted of normal saline only and thus constituted a control.

Experiment G

Dog, weight 10.0 kgm. Intravenous injection of $Al_2(SO_4)_3$, 10 cc. = 1.0 mgm. Al. Given 1 mgm. Al per kilogram.

TIME	STOMACH TOTAL Al	SMALL INTESTINE TOTAL Al	LARGE INTESTINE TOTAL Al	URINE, Al/100 cc.	REMARKS
	mgm.	*mgm.*	*mgm.*	*mgm.*	
9:30					Amytal
11:30					Ether
12:30	0.000	0.000	0.000	0.000	Completion of first washing of loops
12:32					Injection begun
12:58					Injection finished
6:15	0.017	0.050	0.067	2.050	Second washing

Notes: January 10—Dog not fed
January 11—Dog not fed
January 12—Dog given 15 grams $MgSO_4$
January 13—Experiment performed
Total urinary excretion 12:30–6:30 = 30 cc.

Experiment H

Dog, weight 11.1 kgm. Intravenous injection of $AlCl_3$ solution, 10 cc. = 1 mgm. Al. Given 1 mgm. Al per kilogram.

TIME	ALUMINIUM TOTAL			PHOSPHORUS TOTAL			REMARKS
	Stomach	Small intestine	Large intestine	Stomach	Small intestine	Large intestine	
	mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	
Feb. 20							Dog not fed
Feb. 21							15 grams $MgSO_4$
Feb. 22							
7:15							Amytal
10:55	Trace	0.39	0.002	0.19	0.38	0.17	Normal control samples
10:58							Injection begun
11:28							Injection finished
2:10	Trace	1.28*	0.007	1.41	23.2*	1.56	Died. Intestinal tract congested

* Washings contained much blood.

Experiment I

Dog, weight 16.0 kgm. Intravenous injection of $Al(SO_4)_3$, 10 cc. = 1 mgm. Given 1 mgm. per kilogram.

TIME	ALUMINIUM TOTAL			PHOSPHORUS TOTAL			REMARKS
	Stomach	Small intestine	Large intestine	Stomach	Small intestine	Large intestine	
	mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	
Mar. 26							Not fed
Mar. 27							15 grams $MgSO_4$
Mar. 29							
7:15							Amytal
10:30	0.000	0.000	0.032	0.25	0.78	0.27	Loops washed. Control sample
10:35							Injection begun
11:00							Injection finished
3:20	0.026	Lost	0.072	0.95	2.97	0.38	Died. Intestines appeared normal

Experiment J—control experiment

Dog, weight 14.0 kgm. Intravenous injection of physiological saline solution, 10 cc. per kilogram.

TIME	ALUMINIUM TOTAL			PHOSPHORUS TOTAL			REMARKS
	Stomach	Small intestine	Large intestine	Stomach	Small intestine	Large intestine	
	mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	
May 19							Not fed
May 20							Not fed
May 21							15 grams $MgSO_4$
May 22							
7:15							Amytal
9:50	0.000	0.000	0.000	0.70	1.02	1.00	Loops washed. Control samples
9:55							Injection begun
10:40							Injection finished
3:00 p.m.	0.000	0.000	0.000	1.59	3.40	1.19	Died. Intestinal tract appeared normal

Discussion of results. The fate of aluminium after intravenous injection of 1 or 2 mgm. per kilo in the dog is shown in these experiments. Its distribution to the various fluids of the body occurs promptly. Aluminium appears in the bile, lymph and urine in a few minutes after the injection. At the same time the amount in the blood tends to decrease

although fluctuations occur. The rate of disappearance is slow, the aluminium of the blood sometimes maintaining a fairly constant high level for a number of hours.

The rate of disappearance also varies from moment to moment, as does the rate of increase or decrease in the other fluids. This must depend on complex physical fluctuations such as circulatory rate and blood pressure.

The amount of aluminium appearing in the bile, lymph and urine is not always enough to account for that which has disappeared from the blood in the same time interval. This points to storage, temporary or permanent, of some of the injected aluminium. If we may suppose that a dog fasted for a day or so before the experiment is in "aluminium-equilibrium" we might expect that all the aluminium injected could be recovered if the experiment could be prolonged sufficiently.

Experiment F suggests that aluminium may be absorbed from the gastro-intestinal tract by the lymph, rather than through the blood since the blood throughout this experiment did not show aluminium, while the lymph did. We have no normal control sample on this animal, but from the results for the others we may assume that there was only a trace, if any, of aluminium in the normal lymph. Since the lymph from the thoracic duct was diverted this aluminium did not reach the blood. This is only one experiment, but the suggestion is very interesting, and conjectures as to its meaning lead to the question of the relation of aluminium to fat metabolism.

The last group of experiments shows another fact, that aluminium is excreted through the walls of the intestinal tract. These experiments are not to be regarded as quantitative, since exact measurements of the amount of the tissues used, and control time intervals were not attempted. This type of experiment could be made quantitative and information could be gained concerning the rate of the excretion of ions through the walls of the gastro-intestinal tract at various levels.

It is clearly shown that after intravenous injection of 1 mgm. per kilo of aluminium salts, aluminium is excreted through the walls of the stomach, the small and large intestine. This cannot be attributed to contamination, since all parts were carefully washed beforehand, and the bile excluded.

The fact that aluminium is in part eliminated through all portions of the gastro-intestinal tract may account for the difficulty of finding it in large quantities in the blood.

Determination of phosphorus was made because of an idea which has long held the approval of physiologists, although repeatedly discredited by careful investigation. This idea is that aluminium, if absorbed from the intestinal tract, will divert phosphorus from urinary to fecal excretion to form insoluble aluminium-phosphate, thus rendering the phosphorus

"unavailable" for metabolic uses. Modern physiology would see in such a diversion only another manifestation of the body's delicate equilibratory mechanism. It is, as we have seen, not yet proved that aluminium phosphate is formed, or that if formed it is insoluble in the gastro-intestinal tract. But it was desirable to put this idea to a direct test.

A comparison of results shows that, as closely as we may judge from such inexact experiments, as much phosphorus is excreted through the intestinal walls after an intravenous injection of physiological saline as after aluminium solutions. There is here no evidence that phosphorus excretion through the intestinal wall is increased by the presence of aluminium. These two are probably independent of each other. In general it would seem that more aluminium is excreted, per unit of area, through the walls of the small intestine than through the stomach or large intestine. The same seems to be true for phosphorus.

These experiments exemplify the physiological principle that the body maintains an exact ionic balance which may be disturbed for a short time by the sudden addition of an excessive amount of one ion, but the equilibrium with respect to that ion is adjusted promptly by distributing it through the body and by prompt excretion.

One thing worthy of note is the decrease in urinary excretion after the injection of aluminium salts. While we have no data of the normal excretion rate for these animals it is obvious that an excretion of 22.1 cc. in seven hours (expt. B) and 22.7 cc. in six and one-half hours (expt. D) is very low for dogs of this size, especially as 5 or 10 cc. per kilo of fluid had been injected. In some cases no urine was obtained after the injection. This cannot be attributed to low blood pressure as in only one case was there any evidence of shock. In one case when complete anuria occurred, sections of the kidney were made. These showed nothing abnormal. We are therefore left to question the effect of the aluminium injection upon the kidney.

CONCLUSIONS

1. After the intravenous injection of aluminium salts in doses of 1 or 2 mgm. of aluminium per kilo in dogs, the metal appears promptly in the bile, lymph and urine.

2. As far as may be judged, this increase in these fluids does not account for the aluminium which has disappeared from the blood. This points to storage in the tissues.

3. Aluminium is absorbed by the lymph when aluminium-rich food is fed to dogs.

4. Aluminium is excreted through the walls of the gastro-intestinal [illegible] at all levels. It is suggested that this gastro-intestinal eliminat[illegible]

aluminium may account for the failure to demonstrate large quantities of the metal in the blood after ingestion of aluminium-rich food.

5. The aluminium and phosphorus excretion through the walls of the alimentary canal are independent of each other.

BIBLIOGRAPHY

(1) STEEL, M. This Journal, 1911, xxviii, 94.
(2) MYERS, V. C., AND D. B. MORRISON. Journ. Biol. Chem., 1928, lviii, 615.
(3) UNDERHILL, F. P. AND F. I. PETERMAN. This Journal, 1929, xc, 15.
(4) UNDERHILL, F. P. AND F. I. PETERMAN. This Journal, 1929, xc, 1.
(5) BELL, R. D. AND E. A. DOISY. Journ. Biol. Chem., 1920, xliv, 55.

STUDIES IN THE METABOLISM OF ALUMINIUM

V. The Relation of Age to the Amount of Aluminium in Tissues of Dogs

FRANK P. UNDERHILL and F. I. PETERMAN

From the Department of Pharmacology and Toxicology, Yale University, New Haven, Conn.

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That aluminium is found in small, but significant amounts in the tissues of normal dogs can no longer be doubted (1), (2). That it is present in the tissues of normal man is also true, since the blood often contains small amounts of aluminium (3) which has also been found in human autopsy materials (4) (and subsequent paper).

Whether this aluminium has a biological function is a matter for conjecture. It may act as a catalyser as Bertrand (5) suggests. That inorganic ions until recently considered "foreign" to the body may exert a profound influence on the animal economy is now being recognized. Small amounts of manganese have been shown (6) to stimulate the formation of coliagglutinins in goats and diphtheria antitoxin in horses. Other metals in minute amounts have been observed to influence physiological processes.

By one investigator (7) it has been suggested that since aluminium is closely related to iron chemically, its function may be similar to that of iron, and that aluminium may, in a measure, replace the iron in ferruginous proteins and accompany iron in its physiological cycle. The evidence is of the most fragile kind. While iron and aluminium are alike in many of their simple inorganic reactions they show many striking dissimilarities in their behavior in complex ions.

It is conjectured that aluminium in the body may play an essential rôle in reproduction since there have been indications that aluminium is a necessary addition to a purified diet for normal reproduction and rearing of young (8) (9) (10).

In our previous work it was found that the aluminium content of tissues varies a great deal in animals subjected to the same experimental procedure (2). Nothing is known of the history of the ordinary laboratory dog; it is therefore impossible to correlate aluminium content with any life circumstance. It seems probable that aluminium content may bear some relation to age, that is, aluminium may accumulate gradually in the animal body as time and exposure go on.

It was desired to analyse a series of dogs of various ages, to determine the following points:

1. Is aluminium present in the embryo?
2. Is aluminium present in the very young puppy?
3. How does the aluminium content vary with age?

The series reported below is composed of 13 embryos from 4 litters, 16 puppies from 10 litters, and 5 adult dogs. The embryos were taken at autopsy from animals which had been subjected to various experiments

TABLE 1

Aluminium content of normal dog embryos

NUMBER	WEIGHT	SEX	LITTER NUMBER	WHOLE EMBRYO	CARCASS	VISCERA	LIVER	KIDNEY	BRAIN	MUSCLE	LUNG	GALL BLADDER	REMARKS
	grams												
1	5.1		1	None									Very young
2	5.4		1	None									
3	4.4		1	None									
4	5.6		1	None									
5	28.5		2		None	Very small trace							
6	27.5		2		None	Very small trace							
7	25.1		2		None	Very small trace							
8	23.7		2		None	Very small trace							
9	24.1		2		None	Very small trace							
10	10.9	M.	3				None	Possible trace	None	None	Possible trace	None	About half to term
11	96.4	F.	3				None	Possible trace	None	None	None	None	About half to term
12	183.0	F.	4				None	None	0.086	None	None	None	Almost term
13	271.0	M.	4				None	None	0.090	None	None	None	Almost term

which could not, presumably, have influenced the aluminium content of the embryos. The age of the embryos is unknown, but the weight is given as well as an estimate of the stage of development. The puppies, if very young, were born in the laboratory and the age was known. Exact age of older puppies was usually not known. The age of the older dogs was estimated by a veterinary surgeon. Dog 1 was known to be 12 years old.

The animals were subjected to no experimental procedure. Embryos were analyzed at once, as were the very young puppies. Older puppies and adult dogs were kept in the laboratory for a day or two without food to be certain that possible absorption from the alimentary tract would not influence the results. The animals were bled to death under ether anes-

thesia, and blood and tissues treated as described in a previous paper (11). Organs of embryos were usually analysed whole.

RESULTS. *The aluminium content of dog embryos.* Table 1 shows the results for the analyses of 13 embryos. When the whole embryo was used for a single analysis no aluminium was found. When large enough so that carcass and viscera could be analysed separately the body contents were found to contain a trace of aluminium. The source of this is probably the

TABLE 2

The aluminium content of blood and tissues of puppies

Al in milligrams per 100 grams

NUMBER	WEIGHT	SEX	AGE	LIVER	KIDNEY	BRAIN	MUSCLE	LUNG	GALL BLADDER	BLOOD	LITTER
	grams										
1	137.8	F.	Less than 24 hours	0.000	Trace	0.000	0.000	0.000	—	—	2
2	122.0		48 hours	0.000	0.000	0.000	0.000	0.340	—	—	2
3	130.0		48 hours	0.480	0.000	0.000	0.000	0.000	—	—	2
4	244.0		4 days	0.000				0.000	—	0.230	10
5	315.0		5 days	0.000				0.000	—	0.300	10
6	624.0	F.	Approximately 3 weeks	0.000	0.000	0.000	0.160	0.280	0.000	—	1
7	894.0	F.	3 weeks	0.000	0.000	0.000	0.320*	0.130	Trace	Trace	1
8	1,012.0	F.	3 weeks	0.000	0.000	0.000	0.140	0.130	0.000	Trace	1
9	911.0	F.	4 weeks	0.000	0.000	0.040		0.070	0.050	0.000	4
10	705.0		4 weeks	0.000	0.490	Trace		0.120		—	4
11	1,100.0	F.	2 months	0.000	0.100	0.040		0.100	0.000	Trace	7
12	2,600.0	F.	3 months	0.140	0.300	0.130		0.180		0.220	3
13	3,200.0	M.	3 months	0.110	0.220	0.000		0.100		0.090	5
14	3,500.0		3 months	Trace	0.510	0.120		0.250	0.000	0.090	6
15	3,200.0		3 months	0.400		Trace		0.350	0.040	—	8
16	4,200.0		5 months	0.420		0.090		0.220		—	9

* Very fat.

alimentary tract since the principal tissues of embryos of more advanced development rarely contain aluminium, a definite amount being found only in the brain of embryos 12 and 13. It may be concluded that aluminium is not usually present in the dog embryo.

In view of these results we must conclude that whatever function aluminium may have in reproduction it is probably not a necessary constituent of the embryo. These results are contrary to the theory of Bertrand who assumed the aluminium content of embryos to be high, the amount decreasing through life to old age.

The aluminium content of the tissues of puppies. Table 2 shows the aluminium content of the principal tissues of 16 puppies ranging in age from one day to five months. In all 10 litters are represented. In the table animals have been arranged according to age.

It will be seen that in general the tissues of very young puppies contain no aluminium, the exceptions found being liver of puppy 3, lung of puppy 2, and blood of puppies 4 and 5. In view of the consistently negative results for the animals of one to five days of age, these four results may possibly be questioned. All pups older than three weeks have aluminium in the lungs. Aluminium appears in the lungs when it may or may not occur in the other tissues in detectable amounts. This suggests a source for the aluminium which is found, practically always, in the blood of adult dogs, even those which have been fasted for several days. Probably the lung is an important channel for the entry of aluminium into the body.

TABLE 3

The aluminium content of certain organs of old dogs

Al in milligrams per 100 grams

NUMBER	WEIGHT	SEX	APPROXIMATE AGE	LIVER	KIDNEY	BRAIN	LUNG
	kgm.		*years*				
1	17.6	F.	12	1.05	0.162	0.222	1.002
2	13.0	F.	5	0.94	0.106	0.319	2.380
3	8.3	M.	10	0.91	0.000	0.384	0.426
4	16.0	M.	5	0.74	0.205		0.806
5	17.0	M.	15	1.42	0.101		5.150

The aluminium in the lungs must of course come from the dust in the atmosphere.

Obviously, it is not intended to imply that the lung is the only path of entrance of aluminium to the body. On the contrary our earlier papers have shown that aluminium may be absorbed from the alimentary canal. It is quite probable that in the present experiments a portion of the aluminium found in the tissues of the older pups had its origin in the food consumed. Nevertheless the lung as a path of entrance of aluminium to the body must be given due consideration.

While variations occur, the older pups tend to show more aluminium than the younger ones. In a subsequent paper this same tendency will be noted for humans.

Distribution in the various tissues is irregular, suggesting that a state of equilibrium with respect to aluminium has not yet been established. While the series is not large we would seem justified in concluding that though aluminium may be absent from the tissues of very young dogs, it is prac-

tically always present, even if in small amounts, in the tissues of puppies after the third month.

The aluminium content of the tissues of old dogs. Table 3 shows the results of analyses of liver, kidney, brain and lung of five dogs of approximately known age. It will be seen that the most important organ, the liver, contains much more aluminium than in either of the two groups previously discussed. The aluminium content of the lung is probably the most interesting finding, since it confirms what has been suggested before, that a life long source for the aluminium found in the blood may be the aluminium accumulated in the lungs from atmospheric dust. While the variation with age is not absolutely rigid, and as the aluminium content of tissues apparently varies not only with age, but with environment and peculiarities of metabolism (3) comparison of the three groups shows that the tissues of old animals tend to contain more aluminium than the tissues of young animals, which in turn contain more than the tissues of embryos.

One might attempt from these noted tendencies to relate the aluminium content and perhaps the general mineral content of the organism with youth, growth and senility, but much more work is needed before such speculation would bear validity. That there is some such relationship seems indicated by the results reported here.

CONCLUSIONS

The data presented illustrate the following points:

1. Tissues of dog embryos contain very little, if any, aluminium.
2. Tissues of puppies contain more aluminium than embryonic tissues.
3. The lungs of puppies after 5 days always contain detectable quantities of aluminium.
4. Tissues of older animals contain more aluminium than either embryonic or puppy tissues.
5. The lungs of old dogs are very rich in aluminium.
6. It is indicated that there exists a direct relationship between the age of an animal and the quantity of aluminium stored in its tissues.

BIBLIOGRAPHY

(1) Myers, V. C. and D. B. Morrison. Journ. Biol. Chem., 1928, lxxviii, 615.
(2) Underhill, F. P. and F. I. Peterman. This Journal, 1929, xc, 15.
(3) Underhill, F. P. and F. I. Peterman. This Journal, 1929, xc, 40.
(4) Myers, V. C. and J. W. Mull. Journ. Biol. Chem., 1928, lxxviii, 625.
(5) Bertrand, G. Bull. soc. hyg. alimentaire, 1920, viii, 49.
(6) Walbum, L. E. Compt. rend. Soc. Biol., 1921, lxxx, 761.
(7) Balls, A. K. Dissertation, Columbia University, 1920.
(8) Osborne, T. B. and L. B. Mendel. Journ. Biol. Chem., 1913, xv, 311.
(9) Daniels, A. L. and M. K. Hutton. Journ. Biol. Chem., 1925, lxiii, 143.
(10) Mitchell, H. S. and L. Schmidt. Journ. Biol. Chem., 1926, lxx, 471.
(11) Underhill, F. P. and F. I. Peterman. This Journal, 1929, xc, 1.

STUDIES IN THE METABOLISM OF ALUMINIUM

VI. The Occurrence of Aluminium in Human Liver and Kidney

FRANK P. UNDERHILL, F. I. PETERMAN, E. G. GROSS and A. C. KRAUSE

From the Department of Pharmacology and Toxicology, Yale University, New Haven, Conn.

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There are very few reports of the aluminium content of human tissues. Gonnerman (1) (2) presented analyses of a limited number of tissues from animals and man. The large amounts found are probably due to the fact that ashing was carried out in porcelain crucibles, which we have found to give high results. Keilholz (3) shortly afterwards reported no aluminium found in several human tissues analyzed. The method used, that of Atack (4), we have found to give low results. The reasons for this are discussed elsewhere (5). Myers and Mull (6) have recently published some results of analyses of autopsy material. Since we have not tested their method we are not prepared to evaluate their results. However, on theoretical grounds it is quite possible that aluminium may be lost during the manipulations. Moreover their dye is not as sensitive an indicator for aluminium as is alizarine.

That human tissues usually contain small traces of aluminium can no longer be doubted. But the relation between the aluminium content of tissues and age, sex, environment and state of metabolism is still to be determined. In the analyses presented below an indication is given as to the direction such an investigation may take.

The tissues analyzed were obtained through the generous coöperation of pathologists all over the United States. While analyses of only a part of the material sent is presented here, we take this opportunity of thanking all those whose coöperation made this study possible.

The livers and kidneys, (preserved with 95 per cent alcohol, occasionally with formalin), obtained at autopsy, were expressed to us, usually in glass containers. Tissues were dried and analyzed as previously described (5). The preserving fluid was also analyzed, and usually found to contain an inconsiderable amount of aluminium.

Results. The results are tabulated below. Analyses for the aluminium content of the liver from twenty-two human subjects are presented, and for kidneys of twenty-one of these. Ages range from 5 to 73 years. Six widely separated localities are represented.

DISCUSSION. Table 1 is a summary of the analyses for the aluminium content of the liver and kidney. It will be seen that the aluminium content ranges from 0.17 to 1.17 mgm. of aluminium per 100 grams or a total of

TABLE 1

The aluminium content of the human liver and kidney

Al in milligrams per 100 grams

PLACE	AUTOPSY NUMBER	AGE	SEX	DIAGNOSIS	LIVER			KIDNEY		
					Weight	Al/100 grams	Total Al	Weight	Al/100 grams	Total Al
					grams			*grams*		
Boston, Mass.	A-24-58	27	M.	Tuberculous meningitis	1,280	0.31	5.65	300	0.43	1.28
	A-24-59	39	M.	Brain abscess	1,460	0.33	4.85	310	0.23	0.71
	A-24-62	49	M.	Brain tumor, lobar pneumonia	1,850	0.27	5.05	410	0.21	0.83
	A-24-63	38	M.	Pneumonia	1,500	0.41	6.21	210	0.69	1.44
Ann Arbor, Mich.	A-190-AB	44	F.	Diabetes	1,905	1.17	22.27	230*	0.64	1.47*
	A-192-AB	67	M.	Peritonitis	1,330	0.80	10.68	162*	0.74	0.45*
	A-193-AB	70	M.	Cerebro-spinal meningitis	1,630	0.84	13.70	145*	0.87	1.26*
	A-195-AB	Adult	F.	Carcinoma	1,360	0.53	7.17	205*	0.38	0.78*
Birmingham, Ala.		73	M.	Scalp injuries, pneumonia	1,102	0.38	4.24	187	0.78	1.46
Cleveland, Ohio	2914	63	M.†	Pneumonia	1,750	0.30	5.24	350	0.31	1.01
	2915	45	M.†	Pulmonary tuberculosis	1,825	0.21	3.89	450	0.26	1.19
	2917	35	F.	Septicemia	1,800	0.53	9.50	325	0.17	0.44
	2918	21	F.	Pulmonary tuberculosis	1,750	0.21	3.71	300	0.22	0.66
	2919	45	M.	Tuberculous meningitis	1,425	0.21	2.97	325	0.28	0.90
New Haven, Conn.	898	65	M.	Arterio-sclerosis	1,450	0.26	3.77	285	0.19	0.53
	901	46	F.	Carcinoma of stomach	1,510	0.27	4.09	300	0.13	0.39
	908	32	M.	Pulmonary tuberculosis	1,600	0.34	5.40	280	0.20	0.56
	911	5	F.	Acute myocarditis	540	0.19	1.01	105	0.15	0.16
	912	43	M.	Peritonitis	1,700	0.39	6.65	285	0.30	0.85
Augusta, Ga.	24-38	Adult	M.†	Gun shot wounds	1,250	0.72	9.02	365	0.71	2.59
	24-45	21	F.†	Typhoid	1,905	0.60	11.44	385	0.61	2.34
	24-47	45	F.†	Nephritis	1,335	0.17	2.24	230		

* One kidney only. † Negro.

2.24 to 22.27 mgm. for the whole organ, if we except the only child in the series, having a total content of 1.01 mgm. The kidney varies from 0.13 to 0.87 mgm. per 100 grams with a total of 0.39 to 2.59 mgm. if we again

except the child with a value of 0.16. There is no absolute agreement between the amount found in the liver and kidney, but in general a low value is found for the kidney when a low value is present for the liver. The opposite tendency is noted, when the liver value is high the kidney value is also likely to be high. That this is not always the case will appear by reference to no. A-190-AB, in which the highest liver value is found. The kidney value is high but not the highest. In no. 901 the lowest kidney content is accompanied by a low but not the lowest liver content. The only case that can be considered normal is no. 24–38, an adult colored male dying from gun shot wounds. Tissues from this case show what seems to be a moderately high aluminium content for both liver and kidney.

Table 2 shows the distribution of aluminium in the tissues with respect to locality. In this table the average value is given for tissues from particular places. It will be noted that livers of cases from Ann Arbor,

TABLE 2

Distribution of aluminium in liver and kidney related to locality

PLACE	NUMBER OF SPECIMENS	AVERAGE Al IN LIVER PER 100 GRAMS	AVERAGE Al IN KIDNEY PER 100 GRAMS
Boston, Mass.	4	0.33	0.39
Ann Arbor, Mich.	4	0.83	0.66
Birmingham, Ala.	1	0.38	0.78
Cleveland, Ohio	5	0.29	0.24
New Haven, Conn.	5	0.29	0.19
Augusta, Ga.	3	0.50	(2) 0.66
Average		0.43	0.40

Mich., show the highest aluminium content, the kidney from Birmingham, Ala., the highest kidney value, although it is not a fair comparison to include this latter result in a table of averages. The kidneys from Ann Arbor also show a high aluminium content. Tissues from New Haven, Conn., and Cleveland, O., show the lowest aluminium content. Tissues from Boston, Mass., have a moderately low aluminium content.

The consistently high values for tissues from Michigan, and the consistently low ones from Connecticut are worthy of notice, as even cases of approximately the same age, selected from these two groups show this wide variation. (Compare no. A-190-AB with no. 901 and no. 910, and no. A-192-AB with no. 898.) These differences are probably not accidental, and could conceivably be traced to some factor in the environment, such as diet, water supply or type of soil.

When the results are tabulated according to age (see table 3) it is seen that the general tendency is for the aluminium content to increase with

TABLE 3

The relation of aluminium content to age and disease

AGE	SEX	LOCALITY	LIVER Al/100 GRAMS	KIDNEY Al/100 GRAMS	AVERAGE LIVER	AVERAGE KIDNEY	OTHER PERTINENT PATHOLOGIC DETAILS
5	F.	Conn.	0.19	0.15	0.19	0.15	
21	F.	Ohio	0.21	0.22	0.38	0.42	Chronic passive congestion of liver
21	F.*	Ga.	0.60	0.61			Ruptured ectopic hemorrhage
27	M.	Mass.	0.31	0.43			Tuberculosis in liver and kidneys
32	M.	Conn.	0.34	0.20	0.40	0.41	
35	F.	Ohio	0.53	0.17			Cloudy swelling and congestion of liver and kidney
							Acute suppurative nephritis
38	M.	Mass.	0.41	0.69			? Pyelitis
39	M.	Mass.	0.33	0.23			
43	M.	Conn.	0.39	0.30	0.40	0.30	
44	F.	Mich.	1.17	0.64			Fatty liver
45	M.*	Ohio	0.21	0.26			Caseous tuberculosis and congestion of liver
							Bilateral pyelonephritis
45	M.	Ohio	0.21	0.28			Congestion of kidneys
45	F.*	Ga.	0.17				
46	F.	Conn.	0.27	0.13			Anemia
49	M.	Mass.	0.27	0.21			
63	M.*	Ohio	0.30	0.31	0.47	0.41	Cloudy swelling of liver and kidney. Mild chronic interstitial nephritis
65	M.	Conn.	0.26	0.17			Generalized arterio-sclerosis
67	M.	Mich.	0.80	0.74			Passive congestion, atrophy and parenchymatous degeneration of all organs
70	M.	Mich.	0.84	0.87	0.61	0.82	Multiple abscesses of liver. Chronic degenerative parenchymatous nephritis. Arterio-sclerosis, adenoma of kidney
73	M.	Ala.	0.38	0.78			Liver small, moderate chronic passive congestion
Adult	M.*	Ga.	0.72	0.71			All organs sound
Adult	F.	Mich.	0.53	0.38			Generalized arterio-sclerosis, atrophy and parenchymatous degeneration of all organs

* Negro.

advancing age. This trend has been indicated for dogs in a previous paper (7).

When one tries to find a relationship between the state of the tissue analyzed and the aluminium content there is one outstanding result (see table 3). The liver showing much the highest aluminium content (A-190-AB) is described in the autopsy report as a "fatty liver." It has previously been suggested that aluminium may be in some way related to fat metabolism (8). While this single result may be due to other unknown factors it again raises the question of a relation between these two substances.

Five cases in which tuberculous infection was a prominent feature show a low aluminium content for both liver and kidney, the age range being 21 to 45 years, the value for liver 0.21 to 0.34 mgm. per 100 grams and for kidney 0.22 to 0.43 mgm. However, these all came from districts showing low aluminium content. It is interesting that two cases of the same age, from the same locality show the same aluminium content in both liver and kidney (see no. 2915 and no. 2919, Cleveland, O.).

There is no apparent relationship between arterio-sclerosis and aluminium content, since one case (no. 898, New Haven, Conn.) shows a low aluminium content and another (no. A-193-AB, Ann Arbor, Mich.) shows a high aluminium content.

SUMMARY

1. The aluminium content of livers and kidneys from human autopsy material is reported.

2. The range found for liver is from 0.17 to 1.17 mgm. per 100 grams, for kidney from 0.13 to 0.87 mgm. per 100 grams.

3. The significance of some of the findings is discussed.

BIBLIOGRAPHY

(1) Gonnerman, M. Biochem. Zeitschr., 1918, xxviii, 401.
(2) Gonnerman, M. Zeitschr. physiol. Chem., 1920, cxi, 32.
(3) Keilholz, A. Pharm. Weekbl., 1921, lviii, 1482.
(4) Atack, F. W. Journ. Soc. Chem. Ind., 1915, xxxiv, 936.
(5) Underhill, F. P. and F. I. Peterman. This Journal, 1929, xc, 1.
(6) Myers, V. C. and J. W. Mull. Journ. Biol. Chem., 1928, lviii, 625.
(7) Underhill, F. P. and F. I. Peterman. This Journal, 1929, xc, 62.
(8) Underhill, F. P., F. I. Peterman and S. L. Steel. This Journal, 1929, xc, 52.

STUDIES IN THE METABOLISM OF ALUMINIUM

VII. The Aluminium Content of Some Fresh Foods

FRANK P. UNDERHILL, F. I. PETERMAN, E. G. GROSS and A. C. KRAUSE

From the Department of Pharmacology and Toxicology, Yale University, New Haven, Conn.

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The aluminium content of foods has received attention from a number of investigators. Langworthy and Austin (1) published extensive analyses of foodstuffs and natural waters. Kratzman (2) found aluminium distributed in many plants. The work of Stoklasa (3) and his associates is summarized in a recent book. They found aluminium in many plants, but they were more interested in its relation to botanical grouping and function of the parts analyzed than in the amount in the edible portions. Myers and Voegtlin (4) reported that a large percentage of the aluminium found in grains and a few root vegetables was water soluble. Gonnerman (5) reports analyses of various breads and brans. Wolff et al. (6) report a few analyses of eggs.

Knowing that small amounts of aluminium may escape detection by the usual methods of analysis we wished to determine the aluminium content of fresh foods by means of the very sensitive method already described (7).

The materials were purchased in various local markets. They were of the best quality obtainable at the particular season. Usually vegetables and fruits were quite fresh and crisp. They were carefully cleaned, peeled when necessary, dried at 105° and ashed as previously described. The edible portion only was used. Meats were fresh.

Because of the possibility of wide variation in moisture content four analyses were usually carried out on each lot of material. The average figures are given. Variation was usually not large. Results are reported for aluminium in milligrams per 100 grams of fresh edible material.

Results. The tables show the results obtained. It is found that of all the materials analyzed cherries and onions contain by far the largest amount of aluminium. Further, both the Connecticut sour and California sweet cherries contain a large amount of aluminium. The three different lots of onions showed a high aluminium content.

The foods containing between 1 and 2 mgm. of aluminium per 100 grams are lettuce, milk, flour and liver. It will be noted that liver from the calf

TABLE 1

The aluminium content of fresh foods

MATERIAL	DESCRIPTION	NUMBER OF SOURCES	Al/100 GRAMS FRESH	AVERAGE	REMARKS
Beans, string	Varying degrees of ripeness	3	0.36 0.61 0.91	0.63	
Beets	Peeled	3	0.34 0.48 0.61	0.48	
Corn	Sweet Cut from cob	2	0.25 0.26	0.26	
Lettuce	Head	3*	2.50 0.58 0.46	1.18	1st sample very young
Onions	Peeled	3	4.60 3.76 4.59	4.31	
Peas	Shelled	3	0.28 0.32 0.33	0.31	
Potatoes	Peeled	3	0.38 0.32 2.20	0.97	1st sample Florida 2nd sample Connecticut 3rd sample unknown
Apples	Variety unknown Duchess	1 1	0.015 0.08	0.047	
Cantaloupe	Edible portion	2	0.40 1.14	0.77	
Cherries	Pitted	2	3.18 3.80	3.49	Native sour California sweet
Oranges	Peeled	1	0.088	0.088	California seedless
Peaches	Peeled and stoned	2	0.93 0.83	0.88	Free stone Cling stone
Watermelon	Ripe heart	2	0.027 0.028	0.028	

TABLE 1—*Concluded*

MATERIAL	DESCRIPTION	NUMBER OF SOURCES	Al/100 GRAMS FRESH	AVERAGE	REMARKS
Eggs	Without shell	2	0.015		White shelled
			0.019	0.017	Brown shelled
Flour		3	2.30		"Gold Medal"
			1.62		"Pastry"
			1.16	1.69	"Mother's Best"
Liver	Calf	2	1.78		
			1.69	1.73	
Liver	Pig	3	2.07		
			1.70		
			1.54	1.77	
Beef		1	0.50		
Pork		1	0.44		
Mutton		1	0.43		
Milk	Whole	3	1.39		
			1.53		
			1.71	1.54	

TABLE 2

Summary—average aluminium content of foods

Al as milligrams per 100 grams

SUBSTANCE	Al/100 GRAMS	SUBSTANCE	Al/100 GRAMS
Apples	0.047	Liver—pork	1.770
Beans—string	0.630	Mutton	0.430
Beef	0.500	Milk	1.540
Beets	0.480	Onions	4.310
Cantaloupe	0.770	Oranges	0.088
Cherries	3.490	Peaches	0.880
Corn—sweet	0.260	Peas	0.310
Eggs	0.017	Pork	0.440
Flour	1.690	Potatoes	0.970
Lettuce—head	1.180	Watermelon	0.028
Liver—calf	1.730		

and from the pig have practically the same aluminium content. The materials having less than 1 mgm. but more than 0.2 per 100 grams are

string beans, beets, cantaloupe, sweet corn, peaches, pears, potatoes and meat (beef, pork, mutton). The lean meat from three animal sources has very nearly the same aluminium content. The lowest aluminium content was found in apples, eggs, oranges and watermelon, which had less than 0.1 mgm. per 100 grams.

In these plant products we can find no correlation between aluminium content and botanical group. If many more materials had been analyzed this might have been apparent since Stoklasa has found more aluminium in hydrophytic than mesophytic or xerophytic plants.

It is apparent that a diet containing liberal amounts of fresh fruits and vegetables may contain a significant quantity of aluminium. This would perhaps account for the aluminium found at times in the blood of normal man (8).

SUMMARY

Analyses showing the aluminium content of various fresh foods have been presented.

BIBLIOGRAPHY

(1) Langworthy, C. F. and P. T. Austin. The occurrence of aluminium in vegetable products, animal products and natural waters. New York, 1904.
(2) Kratzman, E. Pharm. Post., 1914, xlvii, 101, 109.
(3) Stoklasa, J. Über die Verbreitung des Aluminiums in der Natur. Gustav Fischer, Jena, 1922.
(4) Myers, C. N. and C. Voegtlin. U. S. Public Health Report, 1914, xxix, 1625.
(5) Gonnerman, M. Zeitschr. physiol. Chem., 1918, cii, 78.
(6) Wolff, L. K., N. J. M. Vorstman and P. Schoenmaker. Chemisch. Weekblad., 1923, xx, 193.
(7) Underhill, F. P. and F. I. Peterman. This Journal, 1929, xc, 1.
(8) Underhill, F. P. and F. I. Peterman. This Journal, 1929, xc, 40.

STUDIES IN THE METABOLISM OF ALUMINIUM

VIII. A Note on the Toxic Effects Produced by Subcutaneous Injection of Aluminium Salts[1]

FRANK P. UNDERHILL, F. I. PETERMAN and ANTHONY SPERANDEO

From the Department of Pharmacology and Toxicology, Yale University, New Haven, Conn.

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Pathological changes in animal tissues after the administration of large doses of aluminium salts have been described. Siem (1) using rabbits, cats and dogs, found the lethal dose of sodium aluminium tartrate to be 0.25 to 0.30 gram per kilo, expressed as aluminium oxide (equal to 1.72 to 2.07 grams of the salt). Even larger doses were given, death resulting in 7 to 31 days. The material was given subcutaneously in divided doses over a period of 2 to 4 weeks. How much the tartrate contributed to the effects observed we are unable to say, but it is now well known that the amount of tartaric acid present is sufficient to cause severe toxic symptoms (2) in the animals employed.

Döllken (3) described degenerative changes in the central nervous system after injections of the same material. Leary and Sheib (4) after feeding puppies 100 to 500 mgm. and rats 500 to 3000 mgm. of aluminium daily as aluminium hydroxide, found a slight disturbance of growth in the later periods for those receiving very large doses. Rats examined after 16 weeks showed no macroscopic lesions.

Roth and Voegtlin (5) fed aluminium tartrate and sodium aluminium lactate to rabbits, cats and dogs. Rabbits fed 20 mgm. of aluminium per day died in 2 months, cats fed 40 to 80 mgm. per day died in 3 to 17 days, dogs fed 20 mgm. per day died in 4 months. Symptoms noted were diarrhea (in cats, alternating with constipation), loss of appetite and weight, and albuminuria. Changes noted varied from congestion in the gastrointestinal tract to corrosion and hemorrhagic areas, as well as congestion of liver, kidney and spleen.

Seibert and Wells (6) found pathological changes in the blood elements after the intravenous injection of aluminium salts. The changes noted

[1] Constructed from a thesis presented by Anthony Sperandeo to the Yale School of Medicine in 1925 in partial fulfillment of the requirements for the degree of Doctor of Medicine.

resemble those found in lead poisoning. The spleen was also the site of pathological effects.

Since 1843 a half dozen cases of acute poisoning in man have been reported. All of these occurred after a large single dose of an aluminium salt, usually an alum. Symptoms described are those generally observed after large doses of metallic salts; i.e., frequent vomiting, acceleration of the pulse, tremor, followed by weakness, depression and sometimes coma and death.

The toxicology of aluminium in relation to plants does not concern us here. In general, small doses stimulate while large doses depress growth. The effect of aluminium salts on plants has been extensively studied by Stoklasa (7).

The present study was designed to determine the pathological effects of aluminium salts, uncomplicated by the influence of an organic radical. It is not proposed to detail the toxic symptoms which have been described sufficiently by others.

Rats, guinea pigs and rabbits were used. They were carefully selected from apparently healthy stock and given all care necessary to avoid factors which might complicate the result.

The chloride and sulfate of aluminium in 20 per cent solution in distilled water were used. Injections were made into the subcutaneous tissue of the thigh. Rats were given a single injection, while guinea pigs and rabbits were given divided doses beginning with 3 to 5 grams per kilo as the initial dose, following with 1 gram on alternating days. Doses are expressed as grams of the salt used.

When animals died autopsies were performed as soon as possible. Tissues to be examined were preserved in formaldehyde, and sections were stained with hemotoxylin and eosin. Tissues from 2 rats, 5 guinea pigs and 5 rabbits were examined microscopically.

Dr. H. Gideon Wells has examined the sections, and his description is included. We are greatly indebted to Doctor Wells for his interest and coöperation in this problem.

Results. The single lethal dose for rats is found to be 7 to 8 grams of aluminium chloride per kilo. This dose produces death in 1 to 3 days. The lethal dose for guinea pigs was 5 to 7 grams per kilo, when the initial dose was 3 grams followed by 1 gram on alternating days. This dose produces death in 3 to 7 days. The lethal dose of either the chloride or sulfate in rabbits is 7 to 8 grams per kilo, when the initial dose is 4 to 5 grams followed by 1 gram on alternating days. This dose produces death in 7 to 11 days (see table 1).

The symptoms produced in the three species were similar. When small doses were given, no symptoms were noticed. During the hours immediately following the injection of a large dose there was no deviation from

normal behavior. Loss of appetite was the first symptom noted. Animals soon showed inactivity and depression. They lay quietly in their cages, manifesting no interest in their surroundings. If forced to move they were slow and clumsy.

The gross post mortem examination revealed marked congestion in all the viscera, especially in the mesenteric vessels. The stomach was greatly distended, the mucosa somewhat swollen and containing a few pin point to pinhead sized hemorrhages. In two guinea pigs and one rabbit the intesti-

TABLE 1

Showing dosages and duration of life of animals receiving $AlCl_3$ and $Al_2(SO_4)_3$ subcutaneously

Doses as grams of salt per kilo body weight

ANIMAL	NUMBER	INITIAL DOSE		SUBSEQUENT DOSE	TOTAL	DEATH
		$AlCl_3$	$Al_2(SO_4)_3$			
		grams			*grams*	
Rat	1	7		None	7	3rd day
	2	8		None	8	2nd day
Guinea pig	1	3		1 gram alternate days	5	7th day
	2	3		1 gram alternate days	6	5th day
	3	3		1 gram alternate days	5	8th day
	4	5		1 gram alternate days	7	4th day
	5	3		1 gram alternate days	7	6th day
Rabbit	1	5		1 gram 8th and 10th day	7	10th day
	2	4		1 gram 3rd, 5th, and 8th day	7	8th day
	3		4	1 gram 3rd, 5th, 7th and 9th day	8	10th day
	4		4	1 gram 3rd, 5th, 7th day	7	8th day
	5		4	1 gram 3rd, 5th, 7th and 10th day	8	11th day

nal mucosa contained a few small greyish superficial ulcers, but generally the intestinal tract was free of pathological changes. Intestinal contents were greenish, and contained no blood or mucus.

Livers of rats and guinea pigs were of a deep red color, soft and friable. Livers of rabbits showed extensive tissue change, consisting of congestion and greyish white, sharply defined areas of discoloration, involving the peripheral portion of the lobes and suggesting necrosis. The gall bladder, except for moderate distention, appeared normal.

The kidneys were pale and soft, but showed no gross evidence of parenchymatous change. The renal capsule could be stripped with ease. The

lung and spleen shared in the general congestion. The brain showed no macroscopic change except for a pale color.

The microscopic examination by Doctor Wells is appended.

PATHOLOGICAL REPORT BY DOCTOR WELLS

Rat 1

Liver. Disintegrated along one side, appearance suggesting post mortem digestion from contact with stomach. Elsewhere liver shows numerous foci of necrosis, mostly central. A large vein contains many leucocytes, resembling an infected thrombus, but there is much post mortem change.

Kidney. Much disintegration of the convoluted epithelium, but of so diffuse a character as to suggest that it is at least partly post mortem.

Heart. Shows no definite changes, although there are alterations suggestive of post mortem change.

Brain. No definite changes recognized in the brain.

Rat 2

Liver. Well preserved. Shows no definitely abnormal conditions.

Kidney. Epithelium of convoluted tubules shows numerous areas of necrosis, much vacuolization, many granular casts, and on the whole, marked evidence of acute toxic injury. No changes in glomerules.

Spleen. Contains much blood, but not an abnormal amount. No definitely pathological changes seen.

Lung. Larger bronchi show extensive round cell infiltration of a chronic bronchitis, but the smaller bronchi are free from this change.

Heart. No recognizable changes.

Stomach. Seems normal.

Guinea pig 2

Liver. No distinct focal necrosis, but a few small groups of cells stain intensely with eosin, and there is a great variability in the staining of the liver cells throughout the section.

Kidney. Epithelium of convoluted tubules is generally much swollen. In some there is necrosis and large hyaline casts are numerous, more so than in any of the rabbit kidneys.

Stomach. No changes.

Intestine. No changes.

Guinea pig 3

Liver. Numerous vacuoles suggesting fatty infiltration to a moderate degree. Epithelium swollen, but no definite necrosis.

Kidney. Epithelium of convoluted tubules shows much vacuolization, with occasional granular casts, and occasional areas of calcification in the tubules.

Spleen. Small Malpighian bodies, atrophic pulp, distinctly fibrotic, containing many cells full of brown pigment.

Stomach. Mucous membrane shows numerous areas of necrosis which are sharply limited, and stain brownish as if with blood pigment. There is no inflammatory reaction about them.

Guinea pig 4

Liver. No noticeable changes.

Kidney. Considerable necrosis of the epithelium. Numerous mitotic figures are seen in the renal epithelium. There are occasional granular, but few hyaline casts.

Spleen. Engorged with blood but not abnormally so. No definite pathological changes.

Stomach. No changes.

Guinea pig 5

Liver. Post mortem digestion on one side. No recognizable changes in other portions.

Kidney. No definite changes.

Spleen. Atrophic; pulp, slightly fibrotic, but without pigmentation.

Intestine. Post mortem changes only.

Stomach. Changes seen, apparently post mortem only.

Pancreas. Normal.

Guinea pig 6

Liver. Slight vacuolization and swelling of epithelium but no other changes except post mortem digestion on one side.

Kidney. No distinct pathological changes except slight spontaneous nephritis.

Spleen. No change.

Intestine. Post mortem changes only.

Rabbit 1

Liver. Extensive typical focal necrosis, chiefly in centers of lobules, not all of which are involved. One large hepatic vein surrounded by necrotic tissue contains a thrombus, which does not fill it completely. No inflammatory reaction.

Kidney. Epithelium of some of convoluted tubules is vacuolated; the lumen of nearly all the tubules is obliterated by swelling of the epithelium. There are distinct areas of necrosis in the renal epithelium, but no casts. The glomerular tufts are swollen, engorged, and contain what resemble hyaline thrombi.

Skin. Shows large area of necrosis with edema, without inflammatory reaction. One section shows a necrotic border, beneath which is a growth of young connective tissue. There are also areas of necrosis in the deeper tissue involving especially the muscles.

Stomach. No changes.

Intestine. No changes.

Rabbit 2

Liver. Shows several large areas of focal necrosis, distinctly central, but the greater part of the liver is free from change.

Kidney. Much less change than no. 1, although there is some swelling and vacuolization in the epithelium of the convoluted tubules. The glomerular tufts are not so much swollen but in some places they show a protein exudate between the tuft and the capsule. Many of the glomerular capillaries contain what seem to be hyaline thrombi.

A section of the alimentary tract which is somewhat disintegrated and cut tangentially, so that it cannot be identified, seems to be highly edematous.

Rabbit 3

Liver. No section present.

Kidney. Epithelium of convoluted tubules badly injured; in some tubules there is severe coagulation necrosis, with and without vacuolization of the cells. Many tubules have lost the epithelium completely, and none of the tubules of any kind are normal; even the collecting tubules show casts and free epithelial cells in their lumen. The glomerules are not swollen as in rabbits no. 1 and no. 2, and some of them show shrinkage of the tufts. Neither are hyaline thrombi present. There are no interstitial changes. The necrosis resembles in severity that seen in tartrate kidney.

Stomach. Mucous membrane seems to be somewhat edematous but unless this was removed from the animal immediately after death the change may be artefact. However there is distinct edema between the muscularis mucosa and the circular muscle.

Intestine. Duodenum shows no changes that can be recognized as ante-mortem.

Pancreas. Same as intestine, as to changes.

Rabbit 4

Liver. No necrosis, but the cells in the periphery of each lobule are much larger and paler than those in the center. Can this represent a stage of regeneration after central necrosis? One section shows a coccidia abscess. Fat stain; a few lobules show a small collection of fat in small granules, partly, apparently in the liver cells, and partly in the Kupffer cells.

Kidney. The changes here are less marked than in the three preceding, the alterations being limited to a slight amount of protein precipitate in some of the tubules; swelling with and without vacuolization in part of the convoluted tubules; a few small areas of epithelial necrosis and an occasional small scar of a hemorrhagic nephritis.

Rabbit 5

Kidney. The convoluted tubules show some necrosis; many contain low flattened epithelium, and some are distended with coagulated protein, which in the collecting tubules takes the form of casts. The appearances here suggest a somewhat older process than in no. 1 and no. 2. The glomerules seem to be little affected.

DISCUSSION. The lethal dose of aluminium chloride and aluminium sulfate for rats, guinea pigs and rabbits, varied from 5 to 8 grams per kilo. This dose, given subcutaneously, produced death in 2 to 11 days.

In the pathological results the outstanding features are injury to the liver and kidney, although probably the spleen shares in this, since the spleens in the rats showed congestion with paucity of lymphoid tissue and increased pigmentation. That liver and kidney damage is often excessive is shown by the description of the sections. The principal site of damage in the kidney seems to be the convoluted tubule, although the glomerular tufts are sometimes swollen. In the liver central necrosis and sometimes fatty infiltration are noted.

The findings described here correspond closely with those of Siem (1). He found a slight congestion in the gastro-intestinal tract as well as a finely granular fatty degeneration in the liver. The kidneys showed

congestion, fatty degeneration and sometimes blood extravasations. There were well filled vessels in the glomeruli and degeneration of the epithelium of the descending ends of the convoluted tubules. The lumens were filled with hyaline casts.

SUMMARY

The pathological results of the subcutaneous injection of lethal doses of aluminium chloride and aluminium sulfate, in rats, guinea pigs and rabbits, are described.

BIBLIOGRAPHY

(1) SIEM, P. Dissertation, Dorpat, 1886.
(2) UNDERHILL, F. P. Journ. Biol. Chem., 1912, xii, 115.
(3) DÖLLKEN. Arch. exper. Path. u. Pharm., 1897–98, xxxix–xl, 98.
(4) LEARY, J. T. AND S. H. SHEIB. Journ. Amer. Chem. Soc., 1917, xxxix, 1066.
(5) ROTH, G. B. AND C. VOEGTLIN. Journ. Pharm. Exper. Therap., 1918, viii, 132.
(6) SEIBERT, F. AND H. G. WELLS. Personal communication.
See also E. E. SMITH. Aluminium compounds in foods. Hoeber, 1928, New York.
(7) STOKLASA, J. Über die Verbreitung des Aluminiums in der Natur. G. Fischer, Jena, 1922.

THE EFFECT OF ACIDOSIS IN STRYCHNINE POISONING

W. F. WENNER AND E. W. BLANCHARD

From the Zoölogical Laboratory, State University of Iowa

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It is now a well established fact that a condition of acidosis in an animal results in a lowering of the excitability of the motor nerves. Acute acidosis can lower the excitability of the nervous system to such a degree that coma may result. A number of investigators have found that acids and acid producing substances decrease the violence of tetanic convulsions. In this connection Haldane, Hill and Luck (1923) found that ingestion of large amounts of calcium chloride resulted in a change in the reactions of the blood toward the acid side.

Acid producing salts such as calcium chloride and ammonium chloride were found to be effective in the treatment of infantile tetany (György, 1922; Gamble and Ross, 1923; Anderson and Graham, 1924).

Boyd, Austin and Ducey (1926) found that the life of parathyroidectomized dogs could be prolonged by administering orally 2 per cent solutions of the acid producing salt ammonium chloride. Wenner (1927) carried on further work and showed that complete recovery from the tetany of parathyroid deficiency could be brought about by frequent doses of 5 per cent solution of ammonium chloride.

The convulsions characteristic of strychnine poisoning appear superficially to be similar to those of total parathyroid deficiency. For this reason it seemed not illogical that treatment similar to that proven efficacious when used in other forms of tetany, might alleviate the symptoms of acute strychnine poisoning.

Dogs of various ages and sizes were used in this investigation. An equal number of control dogs were run simultaneously with the experimental animals. All dogs were bled by cardiac puncture and their normal pH and CO_2 capacity determined. Any dog whose blood showed a marked deviation from the normal physiological range of pH, was discarded. Acidosis was produced in the experimental dogs by the administration of uranium nitrate, sodium tartrate, ammonium chloride or carbon dioxide.

In one series of animals the nephro-toxic substances, uranium nitrate and sodium tartrate were given subcutaneously. Seven dogs received 5 mgm. of uranium nitrate per kilo. Two dogs were given approximately 2.5 grams of sodium tartrate per kilo. After three or four days, when the

pH of the blood of these dogs dropped to about 7.2 and the CO_2 capacity to around 22 volumes per cent, the dogs were injected subcutaneously with a lethal dose of strychnine sulphate, previously found to be $\frac{1}{160}$ grain per kilo.

The acidosis produced by uranium nitrate and sodium tartrate almost completely mask the more violent convulsions and tetanus that are associated with strychnine poisoning. Muscle twitching or spasm or slight convulsion from which the animal soon recovers, may appear. In all cases, with one exception, complete recovery occurred within forty-five

TABLE 1

ANIMAL	DATE	TIME	STRYCHNINE GIVEN	pH	CO_2 CAPACITY	REMARKS
	1928		*grains*		*vol. per cent*	
Dog 7 8 kgm.	March 13			7.34		Received 25 mgm. uranium nitrate
	March 17	10:00 a.m.		7.24	38.6	
		3:44 p.m.	$\frac{1}{20}$			
		3:55				Convulsion
		4:00				Recovered
		4:14		7.02	25.5	Apparently normal
Dog 15 16 kgm.	April 20			7.37	45.3	Received 40 grams sodium tartrate
	April 23		$\frac{1}{10}$	7.22	27.3	No strychnine effect noticed
	April 28	2:45 p.m.	$\frac{1}{10}$	7.24	23	
		3:10				Convulsion
		3:14		7.08		
		3:30				Muscle twitching
		3:35				Recovered

minutes. One dog died one hour after the injection of strychnine. In this case death was apparently due to uranium nitrate poisoning. Data of one dog that received uranium nitrate and one that received sodium tartrate are presented in table 1.

The nephrotoxic substances used to bring about a fall in pH and alkali reserve are evidently not in themselves effective in inhibiting the physiological action of strychnine. This fact is adequately demonstrated in the following experiments where NH_4Cl and CO_2 were employed.

One and one-half hours after receiving 100 cc. of a 5 per cent solution of NH_4Cl by stomach tube, the dogs were bled for pH and CO_2 capacity. When these figures were found sufficiently low, the dogs were injected with strychnine sulphate. Data of two of these experimental dogs are summarized in table 2.

Carbon dioxide was administered in the manner described by Swingle, Wenner and Stanley (1928). Dogs were placed in a chamber in which the CO_2 pressure was increased sufficiently to produce unconsciousness. When

TABLE 2

ANIMAL	TIME	STRYCHNINE GIVEN	pH	CO_2 CAPACITY	REMARKS
		grains		*vol. per cent*	
Dog 12 14 kgm.	10:30 a.m.		7.39	46.8	Given 100 cc. 5 per cent solution NH_4Cl by stomach tube
	1:30 p.m.		7.19	21.1	
	2:45	$\frac{7}{80}$			
	2:55 to 3:20				Muscle spasms
	3:23		7.04	17.5	
	3:35				Recovery
Dog 13 20 kgm.	10:00 a.m.		7.4	44.8	Received 100 cc. 5 per cent solution NH_4Cl by stomach tube
	11:30		7.23	21.3	
	2:45 p m.	$\frac{1}{8}$			
	2:55				Slight tremors
	3:03				Violent convulsions
	3:15		7.12		
	3:30				Recovered

TABLE 3

ANIMAL	TIME	STRYCHNINE GIVEN	pH	REMARKS
		grains		
Dog 11 9 kgm.	3:55 p.m.		7.32	Placed in CO_2 chamber
	4:25	$\frac{9}{160}$	7.03	Taken from chamber CO_2 given for 30 minutes
	4:35			Returned to chamber
	5:00			Taken from chamber
	5:45			Spasms
	6:05			CO_2 given for 20 minutes
	6:07		7.14	
	7:00			Normal

this condition occurred the dogs were removed from the chamber, bled, injected with strychnine and then immediately returned to the box.

The length of the recovery period is considerably longer with this

method of producing increased acidity than with the other methods employed. In two cases typical strychnine tetanus and convulsions appeared 45 minutes after removal from the CO_2 chamber. This is probably due to the fact that the lactic acid which increases considerably during asphyxiation soon disappears when a normal amount of oxygen is supplied. Macleod (1921) has shown that the lactic acid of the blood returns to a normal concentration within an hour after asphyxia and anoxemia. However, animals injected with lethal doses of strychnine will recover permanently if kept in the CO_2 chamber for a period of one or more hours. The record of one of these experimental dogs is given in table 3.

TABLE 4

ANIMAL	TIME	STRYCHNINE GIVEN	pH	CO_2 CAPACITY	REMARKS
		grains		*vol. per cent*	
Dog 36	3:00 p.m.		7.38	43.2	
8.75 kgm.	4:10 p.m.	$\frac{1}{20}$			
Control	4:20 p.m.				Violent convulsions
	4:40 p.m.		6.92	18.3	Violent convulsions
	5:20 p.m.				Died in convulsions
Dog 16	10:35 p.m.		7.38	43	Started injecting 5 per cent solution $NaHCO_3$ intravenously
12.5 kgm.	10:46 p.m.	$\frac{1}{10}$			
	10:48 p.m.				$NaHCO_3$ injection stopped. Dog received 7.5 grams $NaHCO_3$
	10:50 p.m.		7.56		
	10:58 p.m.				Violent convulsions
	11:10 p.m.		7.08		Violent convulsions
	11:22 p.m.		7.10		Violent convulsions
	11:24 p.m.				Died in convulsions

It is the initial drop in pH of the blood and not the fall produced by convulsions and the resulting anoxemia that is effective in lessening the activity of strychnine. Although all of the experimental animals did not show the same degree of reflex hyper-excitability, the spasms or convulsions occurring interfered sufficiently with respiration to bring about an increase in lactic acid and a further decrease in pH. Significant increases in the level of the lactic acid of the blood during asphyxiation have been reported by McGinty and Gesell (1925) and Swingle, Wenner and Stanley. In the control dogs where the convulsions were more violent, remarkably low figures for pH and CO_2 capacity were obtained. All control dogs died in convulsions within one hour after receiving strychnine.

An increase in pH of the blood augments strychnine action. Convulsions are more violent and more frequent than in the controls. Death appears sooner.

Some time ago Frey (1922) and Gürber (1922) suggested that alkaloids produce their toxic effect by combining with the body proteins. Experiments *in vitro* by other investigators show that the amount of alkaloid taken up by proteins is controlled by the H ion concentration of the media. Labes (1922) found that tadpoles swam about normally in acid solutions of atropine and cocaine; in basic solutions they died very quickly.

Petrunkin and Petrunkin (1927) in their work on the fixation of alkaloids by gelatin found that the alkaloid was fixed by the gelatin only on the basic side of the isoelectric point. They believe that the characteristic contraction of muscle following administration of veratrine ceases when the lactic acid content of the muscle increases sufficiently to lower the pH of the muscle below the isoelectric point of the tissue proteins. While the muscle is in the resting stage the acid disappears with a corresponding increase in pH. Above the isoelectric point the muscle proteins are again rendered capable of fixing the veratrine with the characteristic veratrine contractions resulting.

In this experiment the low initial pH added to the high degree of acidity produced by the lactic acid formed during the first few muscle spasms may decrease the acidity of the body tissues to a value on the acid side of the isoelectric point of the proteins. This would result in a non-fixation of the alkaloid and a disappearance of the symptoms of strychnine poisoning.

It is accepted at the present time that strychnine affects primarily the central nervous system. Sherrington (1905) has shown that strychnine creates hyper-excitability by changing the process of inhibition to one of excitation. Burridge (1917) states that the action of strychnine is manifested by a facilitation of the passage of the nerve impulse across the synapse.

Zotterman (1920) has shown, using the sciatic-gastrocnemius nerve-muscle preparation, that perfusion with a fluid on the acid side of neutrality causes the nerve ending to conduct with a decrement as shown by an increase in the least time interval necessary for muscular summation. It has been shown (Burridge, 1927) that, when a frog's heart is perfused with a solution more acid than the normal blood, a depressing action results. It would appear from our results that the acidosis in the animal produces an effect opposite to that of strychnine and thus decreases the activity of this alkaloid.

The increased decrement in conduction of the nerve impulse might be brought about by the action of the H ion *per se* or indirectly by the action of the H ion upon the chemical constitution of the nervous system. Burridge (1914, 1917, 1927) has suggested in his experiments on the perfused

heart that inhibition is essentially a decalcifying process and that the positive charge carried by the H ion has a decalcifying effect upon tissues. That the process of excitation is due to calcium has been demonstrated by using perfusing solutions of varying calcium tensions. The higher the calcium tension the less readily did the tissues show inhibition phenomena. Thus from Burridge's work it would appear that the increased acidity of the animal's tissue resulted in a decalcification of the synapses.

The activity of strychnine in creating hyper-excitability by its calcifying action is, in this experiment, probably decreased by the decalcifying effect of the H ions.

SUMMARY

Dogs were given lethal doses of strychnine after they had received various substances which produced a condition of acidosis. The experimental animals recovered. All control dogs died within 1½ hours. With an increase in pH of the blood strychnine action was augmented and death resulted more quickly than in the controls. Possible interpretations are offered in the text.

BIBLIOGRAPHY

ANDERSON, G. H. AND S. GRAHAM. 1924. Quart. Journ. Med., xviii, 62.
BOYD, T. E., W. C. AUSTIN AND E. F. DUCEY. 1926. This Journal, lxxxii, 225.
BURRIDGE, W. 1914. Quart. Journ. Exper. Physiol., vii, 167.
1917. Quart. Journ. Med., x, 157.
1927. Quart Journ. Exper. Physiol., xviii, 1.
FREY, E. 1922. Arch. Exper. Path. u. Pharm., xcv, 36.
GAMBLE, J. L. AND G. S. ROSS. 1925. Amer. Journ. Dis. Child., xxv, 470.
GÜRBER, A. 1922. Arch. Exper. Path. u. Pharm., xcv, 192.
GYORGY, P. 1922. Jahrb. f. Kinderh., xcix, 1.
HALDANE, J. B. S., R. HILL AND J. M. LUCK. 1923. Journ. Physiol., lvii, 301.
LABES, R. 1922. Biochem. Zeitschr., cxxx, 14.
MACLEOD, J. J. R. 1921. This Journal, lv, 184.
MCGINTY, D. A. AND R. GESELL. 1925. This Journal, lxxv, 70.
PETRUNKIN, A. AND M. PETRUNKIN. 1927. Journ. Gen. Physiol., xi, 101.
SHERRINGTON, C. S. 1905. Proc. Roy. Soc. Ser. B, lxxvi, 269.
SWINGLE, W. W., W. F. WENNER AND P. STANLEY. 1928. Physiol. Zoöl., i, 172.
WENNER, W. F. 1927. This Journal, lxxxi, 162.
ZOTTERMAN, Y. 1929. Journ. Physiol., liv, lxxvi.

ACTION OF INSULIN ON THE MOTILITY OF THE GASTRO-INTESTINAL TRACT

I. Action on the Stomach of Normal Fasting Man

J. P. QUIGLEY, V. JOHNSON AND E. I. SOLOMON

From the Physiological Laboratory of the University of Chicago

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It was noted by Bulatao and Carlson (1924) that injection of insulin into normal fasting dogs increased the motility of the stomach and also that subsequent injection of dextrose inhibited this motility. It appeared desirable to extend the investigation of these phenomena.

Methods. The results here reported are those obtained from 45 experiments performed on three normal men and one woman living on a normal diet. The subjects usually fasted about 17 hours before beginning an experiment, but the fasting periods varied between 11 and 44 hours. Gastric motility was recorded with the subject lying on a cot. A triple balloon, introduced by way of the esophagus, was connected to water manometers and inflated with air at an initial pressure of about 6 cm. of water. The technique was similar to that employed by Templeton and Johnson (1929) but differed from it chiefly in that a mass of mercury weighing about 5 grams was tied into the tip of the lowest balloon. It served to carry the lowest balloon into the pyloric portion of the stomach; it could be readily located in fluoroscopic examination and it simplified the process of swallowing the triple balloon. A number of fluoroscopic examinations usually showed the mercury to be in the pyloric canal in close proximity to the pyloric sphincter. The upper balloon was usually just below the cardia. The insulin used in our early experiments was the "Iletin" of Eli Lilly & Co. but Insulin-Mulford was used for the greater part of the investigation.

Usually a control record of approximately 15 minutes' duration was made at the beginning of each experiment. Without interruption of the record insulin was injected and the record continued for 2 to 3 hours. In a few cases insulin was injected about one hour prior to the beginning of the record and the subject continued with his normal activities until the balloon was swallowed and the record begun. The results obtained were much the same with either procedure. An extended investigation of the normal gastric motility of one of the subjects (V. J.) reported by Templeton and

Johnson gave valuable control data and in addition control records were made on each of the subjects at various times.

RESULTS. In our control series we have repeated and extended much of the work of Templeton and Johnson; but our periods of fasting were usually twice the length of theirs. In general we have confirmed the type of records obtained under these conditions. In the fasting stomach during a period of activity of moderate intensity the wave of contraction usually begins in the region of the cardia, and sweeps over the stomach toward the pylorus. It should be emphasized that there is no suggestion of a wave of inhibition preceding the wave of contraction. On the contrary there is frequently (as Templeton and Johnson have noted) a rise in tone immediately preceding the contractions in the lower parts of the stomach. During a period of marked activity we were usually unable to determine whether the movements were of the nature of a descending wave.

One record was obtained from a subject after a 44 hour fast. The activity here is similar to that seen after shorter fasting periods.

Relation of subjective hunger pangs with portion of stomach in activity. From his studies on gastric motility Carlson (personal communication) was inclined to believe that the sensation of hunger pangs was related chiefly to activity in the fundic portion of the stomach. This conclusion was based on visual observations which he made on a man with a gastrostomy. Furthermore he noted that pyloric activity is as great during digestion and vomiting as that occurring in the empty stomach during a hunger period and yet in the former case there is no sensation of a hunger pang. The activity in the fundic region, however, may be much greater during a hunger period than during digestion. On the other hand, Templeton and Johnson (personal communication) studied this relation with the triple balloon method and concluded that sensations of hunger coincided with pyloric activity.

Using the same method we have extended this investigation and our study indicates that the sensation of hunger pangs may coincide with activity in any part of the stomach but is related less frequently to activity in the upper region and most frequently to the lower portion of the stomach. When the sensation is related to activity in one portion of the stomach a succeeding or preceding contraction in another part of the stomach usually does not produce a sensation. That is, when the sensation is related to activity of the upper portion of the stomach, activity in the middle may produce a mild sensation but usually pyloric activity does not. The reversed situation also holds. Assuming that gastric activity gives rise to the hunger sensations, this indicates that the sensation does not depend on the part of the stomach in activity but is related to the *type* of activity occurring. It may also be interpreted as indicating that the nervous mechanism is unable to transmit or perceive impulses in rapid

succession so that the last contraction of a series does not produce the sensation of a hunger pang. On the other hand, when sensation is related to pyloric activity conscious sensation may be the result of summation of stimuli. The portion of the stomach giving rise to the sensation may vary in an individual during an experiment. As was noted also by Carlson for the single balloon there is usually a slight delay (about 2 seconds) between the beginning of the contraction and the hunger pang and again between the termination of the contraction and of the sensation. Frequently there is no delay in the sensation resulting from activity of the pyloric region, but usually the sensation begins just after the beginning of the relaxation period and persists after its termination.

Effect of insulin on the motility of the empty stomach. It was definitely established that the subcutaneous injection of insulin in doses from 12 to 20 units is able to increase gastric motility. In only one case did a smaller dose (8 units) give a positive effect and in this case the subject had fasted 44 hours. The increase in gastric motility induced by insulin appeared in 45 minutes to one and a half hours (average about one hour) following the injection. Usually this activity occurred in the pyloric region prior to the remainder of the stomach. Vigorous activity then persisted without the rest periods so characteristic of normal hunger records as long as the experiment continued, frequently about 3 hours. In one case (fig. 1) the activity was still very marked after 5 hours and 10 minutes. Professor Carlson says this is the longest hunger period in man he has ever seen.

Early in the hunger period there was usually an increase in gastric tone, generally recorded from all three balloons and most frequently from the lower one so that the contractions became type II or type III in character. Such a response was most marked in one subject (A. F.). The high tone usually persisted or was only slightly diminished during the remainder of the experiment. Especially in the pyloric region but sometimes in the middle and less frequently in the cardiac region successive brief periods of partial tetany which we have called "type A contractions" (fig. 3c) were noted.

In our experience the characteristic features of the gastric motility induced by insulin are: 1, prolonged duration of the hunger period (period of several hours) (fig. 1); 2, sudden and sustained increase in tone (fig. 3); 3, type A contractions. Our criteria are not very delicate and the effect must be marked to be considered positive. We consider point 1 as being the most characteristic. With the exception of the prolonged periods of activity insulin does not produce any type of record not induced by simple fasting but in our experience points 2 and 3 occurred so much more frequently as to be considered among the characteristic features. A subject showing type I activity normally may show activity between type II and type III with insulin. The increase in activity under insulin is usually

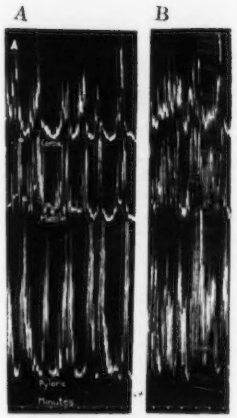

Fig. 1

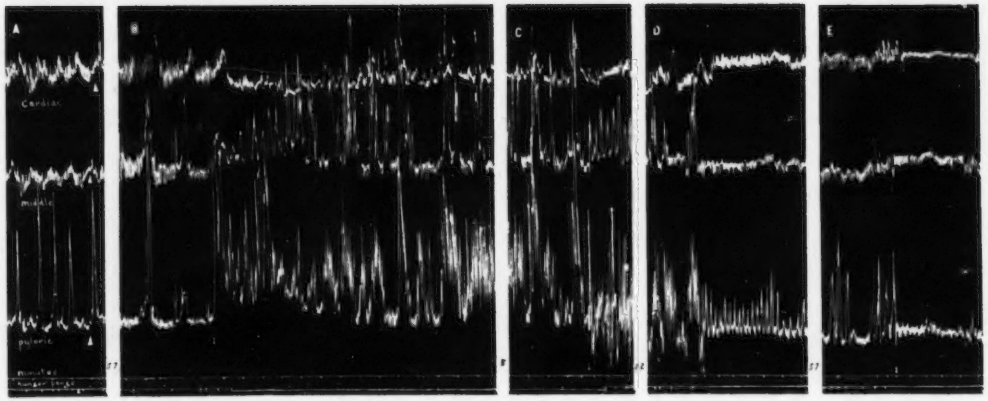

Fig. 2

Fig. 1. Subject fasting 18 hours, given 12 units of insulin; 2 hours and 45 minutes after insulin given 18.5 grams dextrose orally, balloons not collapsed (record not modified); 3 hours and 7 minutes after insulin given 32.5 grams cane sugar orally, balloons not collapsed (record not modified).

A, 3 hours and 58 minutes after insulin (51 minutes after cane sugar); 4 hours and 19 minutes after insulin given 67 grams cane sugar orally, balloons not collapsed (record not modified).

B, 5 hours and 2 minutes after insulin (43 minutes after last cane sugar administration). Record shows the prolonged duration of the hunger period with insulin and also the limited effect of sugar introduced into the stomach.

Fig. 2. Subject fasting 44 hours.

A. Activity at time of injection of 8 units of insulin.

B. *1*, Beginning of typical insulin response 52 minutes after insulin. Subjective sensation of restlessness, continuous uncomfortable burning sensation in region of stomach. Intensity of sensation not related to individual contractions.

C. Sweating, uncomfortable continuous sensation of hunger. *1*, 85 minutes after insulin, introduced 10 grams of dextrose in 100 cc. water into stomach, balloons collapsed for 10 minutes. Uncomfortable sensations diminished.

D. Feeling almost normal. *1*, 109 minutes after insulin, introduced 10 grams dextrose in 100 cc. water into stomach, balloons collapsed 15 minutes.

E. *1*, 170 minutes after insulin, introduced 50 grams dextrose in 150 cc. water into stomach, balloons collapsed 15 minutes. All insulin sensations absent, no return of activity during remainder of experiment (15 minutes).

more marked in the pyloric region than in the remainder of the stomach. This applies more to frequency than to amplitude of the contractions.

The injections of insulin were made at all phases of the motility of the empty stomach; before, during or following a hunger period. From such administration the impression was gained that insulin appreciably shortened the gastric rest period which followed the injection.

The individual contractions after insulin followed each other so rapidly that it has usually been impossible to determine whether they were in the nature of an advancing wave. On occasions, however, it was definitely peristaltic but on other occasions it began in one of the lower balloons before the upper and occasionally waves beginning in the pyloric balloon were noted. This type of movement was not associated with nausea.

Doses of 12 to 20 units usually produced the type of augmentation previously described. In a few cases however, this dose did not yield definite results. The inference that this was sometimes due to delayed absorption from the subcutaneously administered drug is rendered more tenable by the fact that in some of these cases other objective as well as subjective insulin manifestations were absent. There were a few cases however in which hunger, sweating and weakness of a mild degree were not associated with the typical gastric response. The response may have been prevented by psychic inhibition or the state of the subject's sugar reserve, gastric muscles or nervous mechanism. Since the results obtained are related not only to the extent of blood sugar lowering but also to the rate at which this lowering occurs, modifications in the results obtained may be due to variations in these factors. Also by our criteria marked changes must occur to yield results which we have considered typically positive.

Attempts to modify the periods of gastric activity induced by insulin. Several of the procedures known to inhibit the normal motility of the stomach during fasting were tried during a period of activity induced by insulin. It was thus determined that smoking, mild nausea, unpleasant emotions, body discomfort, sight or thought of food, extraneous disturbances, the presence of indifferent substances or food in the mouth or even swallowing food did not modify the activity.

On several occasions dextrose or cane sugar solutions were swallowed or given by stomach tube to the extent of 18 grams dextrose and 100 grams cane sugar in 250 cc. of water without altering the motility during the following one and one-half hours (fig. 1). Subsequent investigation indicated that the solutions had not left the stomach probably because of the balloon in the pyloric antrum and the limited amount of absorption from the stomach. The experiment was then modified by collapsing the balloons for 10 to 15 minutes following the sugar administration. This procedure inhibited partially or completely the gastric motility. Such deflation of the balloons did not lead to gastric inhibition if sugar was not introduced into the stomach.

E. 1, 170 minutes after insulin, introduced 50 grams dextrose in 150 cc. water into stomach, balloons collapsed 15 minutes. All insulin sensations absent, no return of activity during remainder of experiment (15 minutes).

On one occasion adrenalin (0.5 mgm.) was administered subcutaneously and produced a partial inhibition of activity (fig. 4). This inhibition may have been the result of stimulation of the splanchnic gastric nerves, an increased blood sugar, or both.

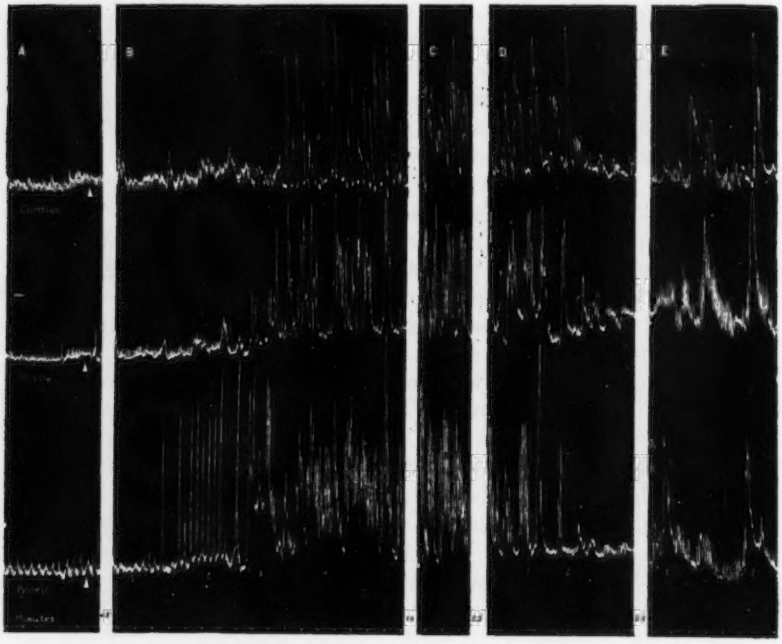

Fig. 3. Subject fasting 19 hours.

A. Injection of 20 units of insulin.

B. *1*. Sensation of hunger became continuous. *2*. Sweating profusely, intensely hungry (sensation continuous), weak and dizzy. *3*, *4*. Sensations same as *2*.

C. Seventy-five minutes after insulin, type A contractions in pyloric and middle portions of stomach.

D. One hundred and three minutes after insulin, intense and continuous hunger, profuse perspiration, malaise. *1*, Injected 1 mgm. atropine sulphate subcutaneously. *2*. Marked decrease in hunger, weakness, perspiration and malaise.

E. One hundred and thirty-eight minutes after insulin, no hunger, mouth dry, no sweating. Weakness and restlessness are as marked as before atropine injection. *1*, *2*. Contractions apparently antiperistaltic (no hunger sensation).

Subcutaneous injection of atropine (0.5 to 1.0 mgm.) was also found inhibit gastric activity for 15 to 30 minutes. The effect was less marked in the mid portion of the stomach than in the remainder of the stom

The atropine probably acted by paralyzing the vagus nerve endings. In some cases the activity following atropine appeared to be antiperistaltic in nature.

Dextrose (30 grams in 200 cc.) warmed to 38° was slowly injected through a tube placed in the duodenum in one experiment. This led to complete inhibition of gastric activity.

During a period of increased gastric activity induced by insulin it was noted that gastric tone was still further increased by sleep.

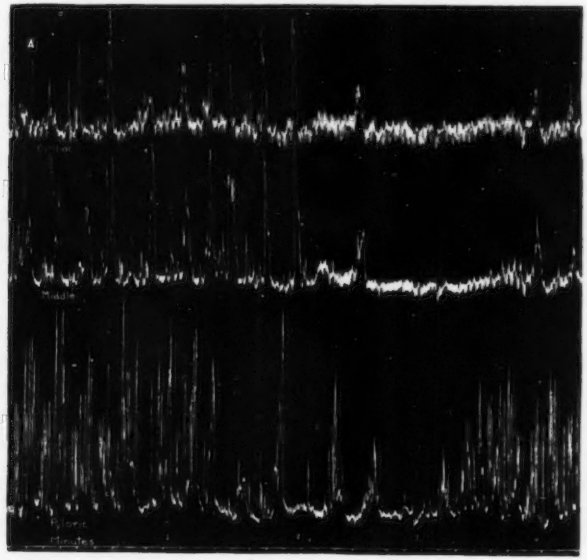

Fig. 4. Subject fasting 16 hours at beginning of experiment.

A. Two hours and 40 minutes after 12 units of insulin: sensations of weakness, marked hunger, restlessness and perspiration. *1.* Injected 0.5 mgm. epinephrine subcutaneously. *2.* No sensation of hunger. *3.* Pounding in head, no sensation of hunger. *4.* No sensation of hunger.

Subjective sensations induced by the experimental procedures. About one hour following the injection of insulin and about 1 to 5 minutes preceding the period of marked gastric activity considered typical of the response to insulin, the subject began to experience a feeling of restlessness and weakness. There was usually an increase in hunger sensations. Sweating usually occurred and isolated contractions became more frequent. As the rise in gastric tone occurred at the first part of the period of activity there was a marked increase in the sensation of hunger. The sensation associated with isolated contractions which had preceded this

period and which could be described as sharp hunger pangs was now replaced by a continuous sensation of hunger and a sense of constriction of the abdominal region so that the subject who had been recording hunger sensations with a key in a series with a signal magnet now felt that he must hold the key closed continuously. The sensation during the brief intervals of gastric rest did not differ from those at the height of a contraction. The sensations were those experienced during the periods of intense gastric activity occurring normally in a fasting person. The sensations were strikingly similar to those described by Carlson (1916) as being typical of hunger, "uncomfortable feeling of tension or pressure and pain referred to the region of the stomach frequently associated with a feeling of general lassitude or weakness. Headache, nausea, nervous irritability, restlessness and vasomotor instability may appear. In persons with stable nervous organization strong hunger may be present without the accessory phenomenon (except nervous hyperexcitability) while in some individuals, the feeling of weakness, headache and restlessness may be so marked as to crowd out the sensation of hunger pangs." These sensations persisted and usually became more marked as the experiment continued and the subject became irritable, ill tempered, and slightly dazed.

These sensations persisted following the termination of the experiment when the balloons were removed and the weakness and mental confusion increased as the subject stood erect and walked about. They did not disappear promptly after eating candy, oranges, or drinking milk (nausea sometimes followed the taking of food) but gradually passed off during the following half-hour.

In those experiments in which sugar was introduced into the stomach and the balloons deflated for 15 minutes alleviation of the "insulin sensations" occurred directly proportional to the decrease in gastric activity seen when the balloons were reinflated. If the motility again returned to that which preceded the sugar administration as it sometimes did when the balloons were collapsed for only ten minutes or the amount of sugar was small, the sensations usually returned to a corresponding degree. Since there was usually complete agreement between gastric motility and hunger sensations, our experiments support the contention that hunger sensations are of peripheral origin and are the direct result of the gastric movements.

In the experiment in which sugar was introduced through a tube in the duodenum, the subject promptly lost all sensation of hunger but became faint and nauseated. It could not be definitely stated whether the gastric inhibition which occurred was related to the irritation of the duodenum or to the increase in available sugar. The latter factor is considered to be chiefly responsible.

When gastric motility was inhibited by the subcutaneous injection of

0.5 mgm. of atropine, all sensations of hunger were absent for 15 minutes but the weakness, uneasiness and restlessness persisted. At the end of this interval there was a partial return of gastric motor activity and also of hunger sensations. With a dose of 1 mgm. of atropine all of the "insulin sensations" but especially the hunger were decreased so that the subject felt "much better." This was followed after about 10 minutes by a disagreeable sensation of discomfort, mental confusion, irritability, decreased ability to think logically and a strong desire to terminate the experiment. This was done and the subject was found to be weak, pale and amnesic. There was no sensation of hunger or thirst. Since there was at the end of the experiment moderately vigorous gastric activity, the absence of hunger sensations at this time may have been related to the disturbed mental condition of the subject; also, some of this activity appeared to be antiperistaltic in nature.

When epinephrine was injected during a period of activity induced by insulin there was a prompt cessation of the sensations of hunger, restlessness and weakness and despite a throbbing in the head, the subject felt much refreshed. This situation persisted for some time but at the end of the experiment (35 minutes after the injection of epinephrine) the subject was weak, nervous and trembling.

Discussion. Insulin may conceivably increase gastric motility by some direct action on the gastric mechanism or it may act indirectly by producing hypoglycemia which then stimulates this mechanism, possibly by reducing the metabolism of carbohydrates. This latter conclusion was favored by Bulatoa and Carlson and our evidence seems to point in the same direction. The action of the stimulatory factor may be directly on the gastric musculature, on the intrinsic gastric nervous system, the myoneural junction or on the central nervous system. The inhibition produced by atropine appears to favor the latter region as the site of action and indicates that the stimulatory impulses are transmitted to the stomach over the vagi. The situation may be similar to the convulsions, central in origin, which are produced by insulin hypoglycemia. Apparently insulin increases gastric motility by an action on the central nervous system and atropine inhibits this activity by its action on the myoneural junction but it must be remembered that atropine will inhibit the movements of strips of the stomach which have been isolated for several days and in which the nervous structures have supposedly ceased to function. The experiments with atropine also show that the gastric activity produced by insulin can be inhibited while the blood sugar remains at a low level.

Since this investigation has established the fact that insulin injections in fasting men give rise to increased gastric motility and parallel with it increased hunger, we may have here a rationale for the use of insulin for the relief of anorexia.

We take pleasure in expressing our appreciation to Doctor Carlson for helpful suggestions and facilities offered. Our thanks are also due to Miss A. Fogelberg and Mr. W. M. Jones who acted as subjects for some of the experiments.

SUMMARY

1. Subcutaneous injection of insulin in amounts of 12 to 20 units usually produces in normal human subjects fasting 11 to 44 hours an increase in gastric motility as recorded by the triple balloon method.

2. The essential features of this response are a, an increase in gastric tone; b, type A contractions; c, a very prolonged hunger period.

3. The gastric hypermotility induced by insulin is not inhibited by smoking, mild nausea, unpleasant emotions, body discomfort, sight or thought of food, extraneous disturbances, swallowing or the presence of indifferent substances or food in the mouth or in the stomach.

4. The gastric motility is inhibited by introduction of dextrose or cane sugar into the duodenum, and also by the subcutaneous injection of atropine or epinephrine.

5. "Insulin sensations," especially hunger, parallel rather closely the degree of gastric activity.

6. Under the experimental conditions described antiperistaltic movements in the stomach were not associated with sensations of nausea.

BIBLIOGRAPHY

BULATAO AND CARLSON. 1924. This Journal, lxviii, 148; lxix, 107.
TEMPLETON AND JOHNSON. 1929. This Journal, lxxxviii, 173.
CARLSON. 1916. The control of hunger in health and disease. Chicago.

ISOSPERMOTOXIN

CHARLES EISEMAN AND MAURICE H. FRIEDMAN

From the Physiological Laboratories of the University of Chicago

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Metalnikoff (1) in 1900 reports having found a toxin in the sera of guinea pigs, of both sexes and of castrates, after having injected them one or more times subcutaneously or intraperitoneally with macerated testes or epididymis. In Guyer's work (2) three rabbits were intravenously injected five times at weekly intervals with semen collected from the vaginae of does after having copulated with three bucks. He found the serum of the animals treated allowed sperm to swim a shorter time than did serum from normal rabbits, and that heating decreased the toxicity of the serum from the treated animals. The next year MacCartney (3) published his results. He macerated rat epididymis and diluted this fluid so as so have 80,000 sperm per cubic centimeter by count. This antigen was injected subcutaneously, on the abdomen, in amounts varying from 0.5 to 1.5 cc. at four day intervals (never, injecting as a total, more than 360,000 sperm into any animal). Testing these animals he found the females sterile for long periods after injection (2 to 22 weeks), that their sera were spermotoxic, and that their vaginal secretions were likewise toxic as compared respectively to normal rat serum and vaginal fluid. Kennedy (4) repeating Metalnikoff's experiments on guinea pigs, injected small amounts (10 cc. of fluid macerate from one testis or epididymis, of which one injection of 0.25 cc. yielded positive results) and found the animals sterile for a period of time and that their sera were toxic to sperm.

These four workers have reported on the guinea pig, rabbit, and rat to the effect that isospermotoxins have been found in the treated animals' sera and that sterility results due to the presence of these immune bodies in the vaginal fluid or to their action on sperm in the body of the males. In 1926 Oslund's (5) results on the intraperitoneal injection of one-fifth of the macerate from a rat testicle into rats, appeared. They were negative in that some of his females were not sterile and that not all of the males' testes showed epithelial change. Fogelson's (6) rats receiving intermuscularly 100,000, 200,000 and 300,000 rat sperm with four day intervals between injections, were found to be sterile for from 6 to 24 weeks, to have precipitins in their sera (in dilution up to 1 to 128) but to have sera not consistently toxic to sperm. Quick (7) introduced suspensions of two

rat testes or epididymides subcutaneously or intraperitoneally into male rats. His findings are to the effect that these treated animals were fertile and that changes found in the testes were due to malposition caused by cysts in the scrotum. Rosenfeld (8) in the same year (1926) administered, as foreign protein, human semen subcutaneously at 3 to 5 day intervals to women. He found no toxin in these patients' sera, though in one case he demonstrated a complement fixation test.

Pommerencke (9) in 1928 reports a large series of well controlled experiments on female rabbits. The antigens were a testis to 10 cc. of Ringer's solution or an epididymis to 15 cc. of Ringer's solution. The controls received suspensions of salivary gland or the vaginal content from a doe after copulation with a vasectomized buck (prostatic, seminal fluid, and vaginal fluid). The injections were made intravenously (a few were intraperitoneal), they were ascending in dosage from 0.2 cc. to 4 cc. at intervals of from 3 to 6 days over a period of 3 to 15 weeks. The results were sterility, for from 6 to 25 weeks, destruction of sperm in the female genital passages, isospermotoxin and iso-agglutinins in the sera, and toxin in the vaginal secretion.

In the experiments here to be reported every animal's serum was tested for isospermotoxin before any antigen was administered. This was done by securing blood by heart puncture, under ether, from rats, guinea pigs, and rabbits,[1] allowing the serum to separate and then incubating 0.1 cc. of this serum with 0.1 cc. of an epididymis macerate in an incubator at 35–37°C. The test was examined periodically by placing a bacteriological loopful on a hanging drop preparation and observing microscopically if the sperm still swam. This time was compared to that of control sera and saline. The incubation was carried out in Wassermann tubes which were stoppered. All antigens were made by macerating epididymides in 0.9 per cent NaCl solution. Testis was treated similarly taking care not to have pieces of tissue larger than would pass through our hypodermic needle.

Our experiments in female rabbits may be divided into four series. The first four females received 5 million rabbit sperm intravenously in one injection. Their sera were tested before and, roughly, at weekly intervals for more than three months. Two other does received similar treatment except that the injections were given semi-weekly, ten in number, and ranged from 65 to 288 million sperm per injection. Still another group of five females received five injections given semi-weekly, and varying from 105 to 97 million sperm per injection. The last series of five does received the macerate from one testicle intraperitoneally (really testis plus epididymis) semi-weekly for five injections. (See protocols.)

The experiments on female rats were performed on ordinary stock white

[1] A few bleedings on the rabbits were by marginal ear vein.

rats and rats from the Wistar Institute. The antigen was from the epididymis and was injected into the hamstring muscles under an ether "rausch." The injections, as per Fogelson (6), were 100,000, 200,000 and 300,000 at four day intervals. The test was run ten days after the last injection on this small series of four females. This experiment was exactly repeated on Wistar females using epididymides from Wistar males. This series was on eight animals. The Wistar rats were kept in strict isolation from the other rats. Following this last experiment the same animals received, 3, 6, 9, 12, 0, and 15 million rat sperm into their hamstring muscles at 3 to 4 day intervals and were tested ten days after the last injection. (See protocol E.)

Guinea pig females, after testing sera, were injected intraperitoneally with the macerate from a whole testicle five times, semi-weekly. Their sera were followed for a month and a half. These injections amounted to 283 milllon sperm each. (See protocol F.)

The results on the rabbits were entirely negative, by our method and that used by Pommerencke (9). In no case did we see any toxic action of the sera of the treated animals over that of normals or saline.

The first group of rat experiments yielded sera from both the treated animals and the controls, which stopped sperm in less time than did the saline. The toxic action was not demonstrable in dilutions less than 1 to 10, or after the sera had been heated to 56°C. for 30 minutes even though complement in the form of non-toxic sera was added. Suspecting Bartonella muris we tested for toxin in sera from Wistar rats; it was not evident. Then splenectomizing our treated "non-Wistar" rats, Doctor Cannon, of the department of pathology, found them infected. The Wistar rats' sera were found to be toxic one week after an intraperitoneal injection of blood from our stock. Hence the work was repeated on Wistar rats in strict isolation. The pre-treatment test revealed some of them to be toxic, however, in dilutions not greater than 1 to 10. The tests, after Fogelson's (6) method was used, and after the series of increased antigen, were just like the first, with treated animals indistinguishable from controls by the test. By splenectomy, of the toxic Wistar rats after our experiments, they were shown to be free of Bartonella infection by Doctor Cannon.

The sera of every treated guinea pig stopped sperm swimming much before the normal guinea pig's serum or saline. The toxic effect was removed by heating to 56°C. for 30 minutes, by dilution greater than 1 to 40, but was demonstrable after such heating if normal guinea pig serum was added. To test the specificity of this antibody we tested it with rabbit and with rat sperm. The sera of treated and control pigs were slightly toxic to them both. This toxicity disappears on heating, and like that of rat serum to rat sperm, did not reappear on adding non-toxic serum (rat serum for rat sperm and rabbit serum for rabbit sperm).

The results of the experiments on the guinea pig females verify the results of Metalnikoff (1) and Kennedy and further prove the toxin produced by the injections, to be antibodies of the group requiring complement for their action, to be specific for guinea pig sperm, and to have been present in concentrations not demonstrable in dilutions greater than 1 to 40. The findings in the experiments on rats are not in accord with those of MacCartney but agree with those of Fogelson. MacCartney's and Fogelson's breeding experiments, besides being contradicted by those of Oslund and Quick, are not conclusive, due to the findings of sterility in "normal" rats at irregular intervals and under ideal conditions by Evans and Bishop (10). MacCartney's reported toxicity in the sera of injected rats might be accounted for by the varying toxicity we found in treated and control rats. In any event, we have not been able to find production of an antibody to rat sperm in female rats' sera when antigen equivalent to his, or far greater that he used, was administered intramuscularly.

The experiments performed on the rabbit does, while equivalent in technique to that of Guyer (2) and Pommerencke (9), yielded consistently negative results and therefore contradictory to their findings. We are skeptical as to the value of Pommerencke's breeding experiments due to our finding breeding records as shown in protocol C and to the irregular sterility reported by Hammond and Marshall (11) on normal rabbits. The divergence in the production of toxicity of the does' sera we cannot explain. We thought that perhaps our females were somehow abnormal. To determine this we tested their ability to produce other antibodies (hemolysins) by the method of Thompson (12). They produced hemolysins to guinea pig corpuscles to a strength of 1 to 10,000. We therefore find our animals normal in this respect.

The data indicate that: 1, it is possible to produce isospermotoxin in the female guinea pig; 2, this toxin requires complement for its action; 3, this toxin does not affect rat or rabbit sperm motility; 4, we have not succeeded by our methods in producing or demonstrating isospermotoxins in rabbit does, and 5, we have been unable by our methods to produce or show an isospermotoxin production in female rats though there is a sporadic, weak toxin present; 6, the toxin found in rats (treated or not) does not withstand a temperature of 56°C. for 30 minutes, and this destruction of its action is not due to lack of complement.

Protocol A. Rabbits (four females). Example of series receiving one injection of 5 million sperm.

8/ 4/27—Bled $\frac{26}{24}$. Test antigen contained 53 million sperm per cc.

8/ 5/27—5 million sperm intravenously

8/ 9/27—Littered 8 healthy young. Bleed $\frac{13+}{11}$

8/15/27—Bled $\frac{11}{?}$
8/20/27—Bled $\frac{10}{11}$
8/26/27—Bled $\frac{12}{11}$
9/26/27—Bred (sperm found in vagina)
10/27/27—Littered
11/28/27—Bled $\frac{17}{15.5}$

Note: $\frac{26}{24}$ indicated that the animal's serum allowed sperm to swim for 26 hours as compared to 24 hours, the time of the saline. Plus indicates sperm swimming when readings were stopped. ? indicating that tube was lost. All test antigens were prepared by using one epididymis to 5 cc. of saline.

Protocol B. (Two rabbit females.) Example of series receiving ten injections varying from 65 to 288 million rabbit sperm intravenously and semi-weekly.

12/ 3/27 Bled
12/ 5 Bled $\frac{8+}{7}$
12/ 6 Received 144 million sperm
12/10 Bled $\frac{10}{10}$
12/12 Received 107.8 million sperm
12/15 Bled $\frac{8}{8}$. Antigen by count contained 110 million per cc. sperm
12/16 110 million sperm
12/19 Bled $\frac{17}{10.5}$
12/20 Received 110 million sperm
12/23 Bled $\frac{18\frac{3}{4}}{8\frac{1}{4}}$. Received 125 million sperm
12/27 Bled $\frac{13\frac{1}{2}}{13\frac{1}{2}}$
12/28 Received 65 million sperm
12/30 Bled $\frac{15+}{11}$
12/31 Received 127 million sperm
1/ 3/28 Bled $\frac{14\frac{3}{4}}{9\frac{3}{4}}$ $\left(\text{Control} = \frac{13\frac{3}{4}}{9\frac{3}{4}}\right)$
1/ 4/28 Received 121 million sperm
1/ 6/28 Bled $\frac{11}{7}$ $\left(\text{Control} = \frac{11}{7}\right)$
1/ 7/28 Received 127 million sperm
1/11/28 Bled $\frac{8\frac{1}{2}}{6\frac{1}{2}}$ $\left(\text{Control} = \frac{8\frac{1}{2}}{6\frac{1}{2}}\right)$
1/12/28 Received 138 million sperm
1/16/28 Bled $\frac{11\frac{1}{2}}{14\frac{1}{2}}$ $\left(\text{Control} = \frac{14\frac{1}{2}}{14\frac{1}{2}}\right)$
1/20/28 Bled $\frac{8+}{3+}$ $\left(\text{Control} = \frac{8+}{3+}\right)$

1/22/28 Copulated with buck C (found sperm in vagina)
2/24/28 Littered
4/28, 29, 30,—5/1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18. On these days received 1 cc. of a 10 per cent solution of washed guinea pig erythrocytes in saline intravenously (as advised by Kolmer)
5/27/28 Bled—found hemolysins in dilution of 1 to 10,000

Note: Test antigens made by using one epididymis to 5 cc. saline.

Protocol C. (Five female rabbits.) Examples of series receiving five intravenous injections of 105 to 97 million rabbit sperm intravenously at semi-weekly intervals.

12/12/27 Copulated with buck D (sperm found in vagina)
2/ 1/28 Copulated with buck D (sperm found in vagina)
2/10 Bled $\frac{6+}{2}$
2/11 Received 100 million sperm
2/15 Received 100 million sperm
2/18 Received 105 million sperm
2/22 Received 101 million sperm
2/25 Received 97 million sperm
3/10 Bled $\frac{7+}{?}$ $\left(\text{Control} = \frac{7+}{?}\right)$
Copulated with buck C (sperm found in vagina)
3/19 Bled $\frac{19+}{7+}$ $\left(\text{Control} = \frac{19+}{7+}\right)$ got identical figures by Pommerencke's method
4/10 Littered
4/28, 29, 30,—5/1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18. On these days received 1 cc. of a 10 per cent solution of washed guinea pig erythrocytes in saline intravenously (as advised by Kolmer)
5/27 Bled—found hemolysins in dilution of 1 to 10,000

Note: Bucks C and D were fertile as proven by use with other dose. Yet D failed twice to impregnate this female before treatment began. Antigens for tests were made by using one epididymis to 5 cc. of saline.

Protocol D. (Rabbits—five females.) Example of series receiving five injections intraperitoneally of the macerate of one testicle including the epididymis semi-weekly.

4/21/28 Bled $\frac{9\frac{1}{2}+}{9\frac{1}{2}+}$. Antigen contained 160 million sperm per cc.
Received 400 million sperm plus debris
4/25/28 Received 264 million sperm plus debris
4/28/28 Received 286 million sperm plus debris
5/ 2/28 Received 217 million sperm plus debris
5/ 5/28 Received the macerate of one whole testicle (no count made)
5/19/28 Bled $\frac{4\frac{1}{2}+}{4\frac{1}{2}+}$ $\left(\text{Control}\frac{4\frac{1}{2}+}{4\frac{1}{2}+}\right)$

Note: Antigens for tests were made from one epididymis to 5 cc. of saline.

Protocol E. Wistar female rats (eight animals). Example of series receiving rat sperm injections into the ham string muscles per Fogelson, followed by the massive doses of antigen.

3/25/28 Bled $\frac{6\frac{1}{2}}{6\frac{1}{2}}$ using sera diluted 1 to 10
Received 100,000 sperm
3/28/28 Received 200,000 sperm
3/31/28 Received 300,000 sperm
4/10/28 Bled $\frac{2+}{2+}$ $\left(\text{Control} = \frac{2+}{2+}\right)$ using sera diluted 1 to 10
4/25/28 Received 3 million sperm
4/28/28 Received 6 million sperm
5/ 2/28 Received 9 million sperm
5/ 5/28 Received 12 million sperm
5/12/28 Received 15 million sperm
5/21/28 Bled $\frac{4\frac{1}{2}+}{4\frac{1}{2}+}$ $\left(\text{Control} = \frac{4\frac{1}{2}+}{4\frac{1}{2}+}\right)$ using sera diluted 1 to 10. Antigen contained 34 million sperm per cc.
6/ 2/28 Splenectomy—in observation period showed no characteristics of Bartonella muris infection

Note: Test antigen was prepared from one epididymis to 5 cc. of saline.

Protocol F. (Five guinea pig females.) Example of the series receiving five semi-weekly injections of testicle macerate intraperitoneally.

2/27/28 Bled $\frac{8+}{8+}$ $\left(\text{Control} = \frac{8+}{8+}\right)$
2/29/29 Received macerate of one testicle containing 283 million sperm
3/ 3/29 Received macerate of one testicle
3/ 7/28 Received macerate of one testicle
3/10/28 Received macerate of one testicle
3/14/28 Received macerate of one testicle
3/26/28 Bled $\frac{\frac{1}{2}}{?}$ $\left(\text{Control } \frac{9+}{?}.\right)$ See test records I, II, III
4/ 8/28 Bled $\frac{\frac{1}{2}}{?}$ $\left(\text{Control} = \frac{5+}{?}.\right)$ See test records IV

Test Record I (3/26/28)

TIME	CONTROLS		INJECTED ANIMALS				
	Guinea pig numbe·						
	77	78	90	94	95	96	97
1:50 p.m.	4+	4+	4+	4+	4+	4+	4+*
2:15 p.m.	4+	4+	+	±	−	±	−
3:05 p.m.			−	−	−	−	−
5:20 p.m.	3+	3+	Note: Test antigen sperm were identical in test I, II, and III				
11:00 p.m.	2+	2+	Similar test run using sperm whose count was 28 million per cubic centimeter				

* Time of incubation.

Test Record II (3/26/28)

(All sera held at 56°C. for 30 minutes)

TIME	CONTROLS		INJECTED ANIMALS				
	Guinea pig number						
	77	78	90	94	95	96	97
1:50 p.m.	4+	4+	4+	4+	4+	4+	4+*
2:25 p.m.	4+	4+	4+	4+	4+	4+	4+
3:10 p.m.	4+	4+	4+	4+	4+	4+	4+

* Time of incubation.

Test Record III (3/26/28)

(All sera held at 56°C. for 30 minutes, every tube then receiving 0.1 cc. of unheated serum of no. 77)

TIME	CONTROLS		INJECTED ANIMALS				
	Guinea pig number						
	77	78	90	94	95	96	7
1:50 p.m.	4+	4+	4+	4+	4+	4+	4+*
2:25 p.m.	4+	4+	±	±	±	±	±
3:00 p.m.	3+	3+	−	−	−	−	−

* Time of incubation.

BIBLIOGRAPHY

(1) METALNIKOFF. Ann. de L'Inst. Pasteur, 1900, ix, 585.
(2) GUYER. Journ. Exper. Zoöl., 1922, xxxv, 207.
(3) MACCARTNEY. This Journal, 1923, lxiii, 207; lxvi, 404.
(4) KENNEDY. Quart. Journ. Exper. Physiol., 1924, xiv, 279.
(5) OSLUND. Journ. Amer. Med. Assoc., 1926, lxxxvi, 1755.
(6) FOGELSON. Surg., Gynec. and Obstet., 1926, xlii, 374.
(7) QUICK. This Journal, 1926, lxxvii, 51.
(8) ROSENFELD. Amer. Journ. Obstet. and Gynec., 1926, xlii, 385.
(9) POMMERENCKE. Physiol. Zoöl., 1928, i, 97.
(10) EVANS AND BISHOP. Journ. Met. Res., 1922, i, 319.
(11) HAMMOND AND MARSHALL. Reproduction in the rabbit. London, 1925.
(12) KOLMER. Infection, immunity and biologic therapy. 3rd ed., 1924, p. 401.

CUTANEOUS RESPIRATION IN MAN

I. Factors Affecting the Rate of Carbon Dioxide Elimination and Oxygen Absorption

LOUIS A. SHAW, ANNE C. MESSER and SOMA WEISS

From the Department of Physiology, Harvard School of Public Health, and the Thorndike Memorial Laboratory of the Boston City Hospital, Boston

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In 1851, Gerlach (1) demonstrated that the surface of the skin of animals and man not only eliminated carbon dioxide, but also absorbed oxygen from the surrounding air. With a simple but ingenious method he attempted to measure quantitatively the amount of carbon dioxide given out and oxygen absorbed over localized skin areas. He estimated that in a normal resting man 4.3 liters of carbon dioxide leave and 1.9 liters of oxygen enter the blood stream through the skin in 24 hours. Gerlach was the first investigator who attributed a respiratory function to the skin. Aubert (2) placed his subjects in a chamber with the neck protruding through an air-tight collar. The carbon dioxide content of the circulating air through the chamber was measured by Pettenkofer's method. Aubert observed that with a rise in the temperature of the air surrounding the skin there was also an increase in the carbon dioxide output. He concluded that one-half of one per cent of the expired carbon dioxide is eliminated through the skin.

Schierbeck (3), working in Rubner's laboratory with a method somewhat similar to that used by Aubert, observed no change in the carbon dioxide output between 29° and 33°C. When the temperature of the chamber was raised over 33°C. a rapid increase in the carbon dioxide output was observed. According to Schierbeck there is a critical temperature of 33°C., below which the carbon dioxide output is slight and independent of a falling temperature, and above which the carbon dioxide output is proportional to the rise of temperature. This conception of the critical temperature of carbon dioxide elimination through the skin became accepted in the literature (4), (5).

Zuelzer (6) investigated the capacity of the human skin to absorb oxygen from gas mixtures rich in oxygen. His method consisted in placing the hand and the forearm in a glass cylinder through a rubber cuff. The cylinder, placed in a water bath, communicated with a bottle filled with oxygen and nitrogen mixtures of known concentration. The analysis of

the gas mixtures was performed by the method of Durig and Zuntz. Zuelzer found that when the skin is surrounded with a high concentration of oxygen, 1.08 to 12.25 cc. of oxygen per minute are absorbed by the skin surface of the entire body. Marked individual variations were noted. Considering that the difference in the tension between the surrounding oxygen and the oxygen content of the blood was 5.3 times higher than under physiological conditions, it follows that 0.2 to 2.3 cc. of oxygen are absorbed by the skin per minute from the surrounding atmospheric air. The average amount of oxygen absorbed by the skin corresponds to $\frac{1}{100}$ of the amount absorbed by the lungs.

Barratt (7) studied the elimination of carbon dioxide from the skin by inclosing the arm in a cylinder. A current of CO_2-free air was passed through the cylinder and the carbon dioxide of the out-going air was collected by suitable absorbents and determined gravimetrically. He found that the carbon dioxide output per hour, when the air surrounding the skin was 35°C., was 95 per cent greater than at a temperature of 25°C. He also found conspicuous daily changes in the rate of carbon dioxide excretion.

Because of the marked variations and conflicting results of previous investigators, we have undertaken to ascertain some of the factors affecting the rate of carbon dioxide excretion through the skin, and to correlate the carbon dioxide output with the oxygen absorption.

METHOD. The subject lay on a couch in a comfortable position with the entire arm in a plethysmograph. The experiment lasted for four hours. Samples of gas were withdrawn every hour from the plethysmograph directly into gas collecting tubes and analyzed in a Haldane apparatus. The change in percentage composition of the carbon dioxide and oxygen multiplied by the total volume of gas in contact with the skin gives the volume of carbon dioxide and oxygen which has been lost or gained over a given interval.

The plethysmograph (fig. 1) is a brass cylinder just large enough to hold the arm throughout its full length. The plethysmograph is sealed at one end, and at the other end is left open for the insertion of the arm. Over the open end a rubber dam or armlet, 1 mm. in thickness, is stretched, through which a hole is cut of such dimensions as to close in snugly about the arm. In order to insure an air-tight contact between the rubber and the skin, a coating of vaseline is applied to the arm and to the margin of the arm-hole in the dam.

When the plethysmograph is filled with room air an inward leak is of no consequence. On the other hand, when the plethysmograph is filled with a gas having a composition different from air, a very small inward leak would invalidate the results. If, for example, the plethysmograph were filled with nitrogen, a slight leak of air into the plethysmograph would

obviously give misleading results with respect to the oxygen which, under these circumstances, would pass from the blood into the gas surrounding the arm. To avoid such a situation a second armlet, similar to that just described, was applied to the plethysmograph in such a manner as to constitute a gaseous seal interposed between the air and the gas in the plethysmograph (fig. 2). Through the space that intervenes between the two armlets, a gas of the same composition as that in the plethysmograph was kept in circulation by passing it from one spirometer (capacity 5 liters) to another. If, then, a small quantity of air should pass through the outer

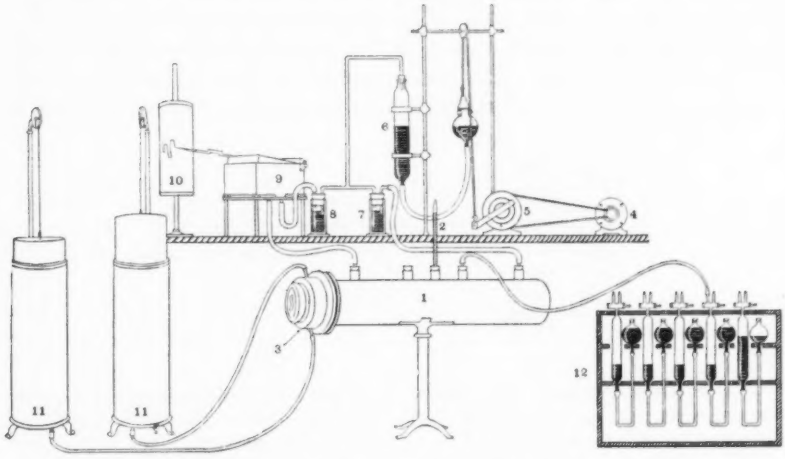

Fig. 1. Apparatus for determining gaseous exchange through the skin of the arm. *1*, plethysmograph; *2*, thermometer; *3*, gas seal; *4*, motor; *5*, crank shaft; *6*, mercury pump; *7*, inlet valve; *8*, outlet valve; *9*, mercury spirometer; *10*, kymograph; *11*, mixing spirometers; *12*, gas collecting tubes.

armlet it at once becomes so diluted by admixture with the gas in the seal that its composition is but slightly affected. In this manner a leak past the inner armlet will only permit the passage of a gas which is so similar in composition to that in the plethysmograph that the effect of the leak is negligible. The presence of the gas seal also greatly reduces any tendency for the gases to diffuse through the rubber armlet and thus disturb the concentration of carbon dioxide and oxygen in the plethysmograph.

It is necessary to maintain the pressure of the gas in the plethysmograph approximately equal to that of the room, otherwise leaks would be unavoidable. The volume of gas in the plethysmograph varies with the changes in temperature and is diminished each time a sample is withdrawn. In order

to maintain the gas in the plethysmograph at atmospheric pressure, a Krogh spirometer was connected to a side tube. The spirometer (capacity 1 liter) has a mercury seal to prevent the absorption of carbon dioxide which would otherwise take place in a water seal. The volume of gas in the spirometer is indicated by the distance of the writing point above the base line as shown on the smoked surface of the kymograph sheet. A shift in the level of the writing point also serves as a means of detecting the presence of a leak.

The gas in the plethysmograph is kept in constant circulation by means of a mercury pump which withdraws the gas from one end of the plethysmograph and delivers it at the other end at a rate of 750 cc. per minute, which is sufficiently great to insure the homogeneity of the gas throughout the system at all times. The pump consists of a mercury levelling bulb which is mechanically raised and lowered, first drawing air through a

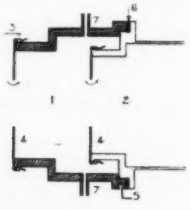

Fig. 2

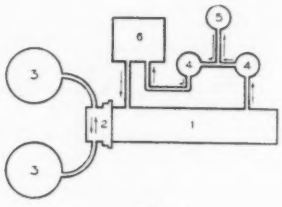

Fig. 3

Fig. 2. Sagittal section of the gas seal. *1*, gas seal; *2*, plethysmograph; *3*, wire binding rubber; *4*, rubber dam; *5*, threaded joint between gas seal and plethysmograph; *6*, rubber washer; *7*, tubes connecting mixing spirometers.

Fig. 3. Schematic representation of apparatus. *1*, plethysmograph; *2*, gas seal; *3*, mixing spirometers; *4*, mercury valves; *5*, mercury pump; *6*, mercury spirometer.

mercury valve into a tube of 250 cc. capacity and then expelling it through a second valve of similar construction. Their operation will be apparent from inspection of figures 1 and 3.

All the experiments were carried out upon two subjects. Experimentation upon a greater number of subjects would have involved individual variations which would render certain comparisons impossible. Subject T. A. was a man twenty-five years of age, weighing 146 pounds; and subject F. K. was a man twenty-six years of age, weighing 130 pounds.

The experiments were carried out in a room in which the temperature could be held constant at any desired level. The temperature of the gas in the plethysmograph was controlled by regulating the temperature of the room. The ratio of the temperature in the room to that of the gas around the arm was thus always held constant. We feared that if the temperature of the gas in contact with the skin was controlled locally

while the temperature of the room was permitted to vary, the temperature effect upon the body as a whole might break through and influence the local temperature effect upon the arm, and thereby introduce a confusing factor.

The analytical error for the determination of carbon dioxide in our system was 0.5 cc. and for oxygen 1.0 cc. In order that the rate of gas diffusion through the slightly varying areas of skin confined by the plethysmo-

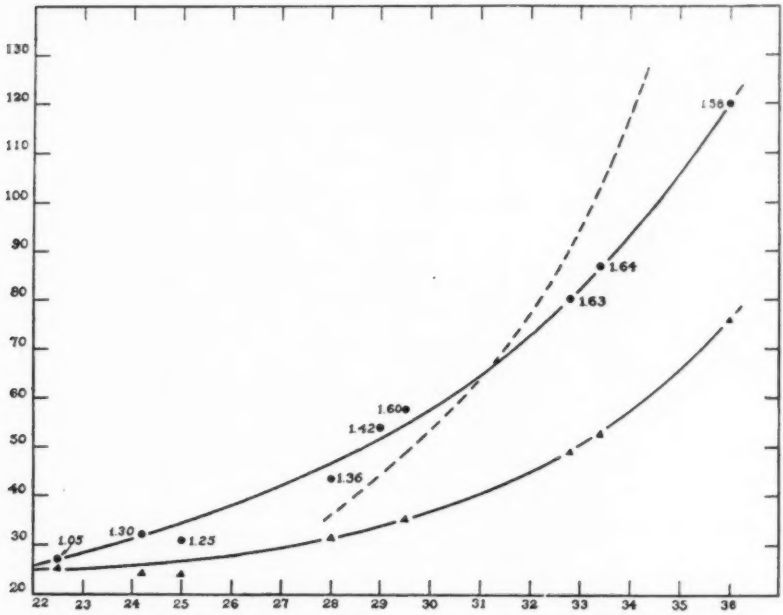

Fig. 4. Cutaneous respiration of T. A. Ordinate, cubic centimeters per hour per square meter of skin surface; abscissa, temperature degrees Centigrade. ●, CO_2 eliminated; ▲, O_2 absorbed; ----------, CO_2 curve transposed from figure 5. Respiratory quotients are given on the curves.

graph may be made comparable, all quantities were converted to cubic centimeters per square meter of skin surface. By the use of tables given by DuBois (8) we calculated that the area of skin confined by the plethysmograph was 0.128 square meter for T. A. and 0.123 for F. K. Therefore, we must multiply the rate of diffusion by 7.81 and 8.13, respectively, in order to make the necessary conversion; and the error, though proportionately the same, becomes about ±4 cc. for carbon dioxide and ±8 cc. for oxygen. Although gas samples for carbon dioxide and oxygen

determination were withdrawn from the plethysmograph every hour as a check upon the accuracy of our work, the values given here represent the difference in the concentration of carbon dioxide and oxygen found to exist between the first and last samples. This value, divided by the duration of the experiment in hours, gave the average value per hour. The error as calculated applies to the total gases measured over an average 3-hour period; therefore, the error for 1 hour will be one-third as great or 1.3 cc. for carbon dioxide and 2.6 cc. for oxygen.

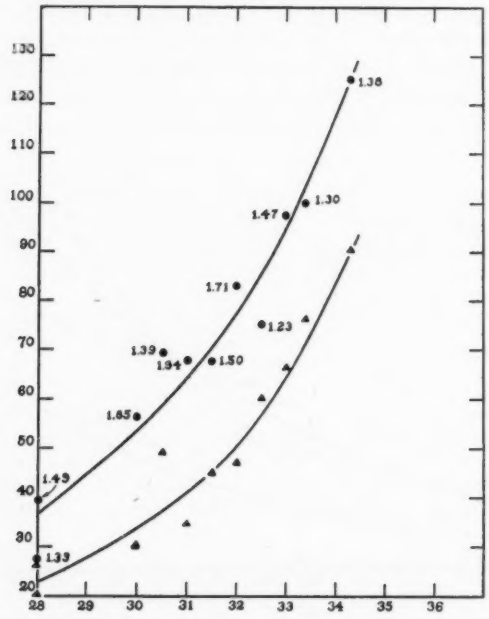

Fig. 5. Cutaneous respiration of F. K. Ordinate, cubic centimeters per hour per square meter of skin surface; abscissa, temperature degrees Centigrade. ●, CO_2 eliminated; ▲, O_2 absorbed. Respiratory quotients are given on the curves.

EXPERIMENTAL RESULTS. *Exchange of carbon dioxide and oxygen through the skin when exposed to air of normal composition.* Figures 4 and 5 give the quantities of carbon dioxide and oxygen which pass through the skin of the arms of two subjects, T. A. and F. K., calculated in cubic centimeters per hour per square meter of skin surface. When the experiments are arranged in order of increasing temperature it will be observed that the rate of diffusion increases as the temperature of the air in contact with the skin increases.

The rate of diffusion in T. A. is different from that of F. K. In order to show the individual variation graphically, the curve for the carbon dioxide excretion of F. K., as given in figure 5, has been transferred to figure 4 so that it may be compared with the curve for T. A.

There is also a seasonal variation in the rate of carbon dioxide excretion which is made apparent in table 1. Corresponding data on the rate of oxygen absorption are not available. When the experiments are arranged

TABLE 1

*Cutaneous respiraton of T. A., showing seasonal variations in rate of carbon dioxide excretion**

EXPERIMENT	DATE	TEMPERATURE IN PLETHYSMOGRAPH	CO_2 EXCRETED	O_2 ABSORBED	$\frac{CO_2}{O_2}$
		°C.	cc.	cc.	
24	September 25, 1928	32.5	53.4	39.1	1.36
25	September 27, 1928	32.0	78.9	73.4	1.07
26	October 2, 1928	32.0	53.9	49.3	1.09
27	October 4, 1928	32.3	55.1	52.8	1.04
28	October 9, 1928	32.5	51.3	39.4	1.30
Average		32.2	58.5		
29	October 11, 1928	32.4	66.3	49.9	1.33
30	October 16, 1928	33.0	76.3		
31	October 18, 1928	31.6	57.7		
33	October 24, 1928	31.4	59.4		
34	October 26, 1928	31.8	71.1		
Average		32.0	66.1		
48	January 8, 1929	32.8	80.3		
50	January 15, 1929	32.8	107.6		
51	January 17, 1929	31.9	69.0		
52	January 22, 1929	32.4	84.7		
Average		32.4	85.4		1.20

* Calculated in cubic centimeters per hour per square meter of skin surface.

in order of the dates and then subdivided into three groups, and the average rate of carbon dioxide excretion taken for each group, we find that the rate of carbon dioxide excretion is 58.5 cc. from September 25 to October 9, 66.1 cc. from October 11 to October 26, and 85.7 cc. from January 8 to January 22, which represents a 46 per cent increase during a period of four months. These experiments were all done upon the same subject, and the average temperature for the experiments of each group was identical; therefore, neither the individual variation nor the temperature have been

instrumental in the progressive rise in the rate of carbon dioxide excretion that took place between September 25 and January 22.

In each of the seasonal groups as given in table 1 will be found variations which cannot be accounted for either by the experimental error (1.33 cc.), by temperature effect, by seasonal variations, or by variations inherent in different individuals, since each of these factors has been controlled. We must, therefore, look to some factor or group of factors other than temperature, season, or individual differences, which is responsible for changes as great as ±14 per cent (expts. 48 and 50) and ±19 per cent (expts. 24 and 25).

The variations in respiratory quotients, as shown in twenty-six experiments, are very marked. If the maximum possible experimental error is added to the low respiratory quotients and deducted from the high respiratory quotients, instead of variations which range from 1.04 to 1.94, the range will be from 1.12 to 1.76, a variation from the average of 1.37, which is still very great, and which we do not attempt to explain.

In calculating the probable rate of carbon dioxide excretion per hour for the entire body under ordinary living conditions, we must take into account the skin temperature. We did not record the skin temperature in our experiments, but it seemed to us that the arm felt most comfortable when the temperature of the surrounding air was about 32°C., and this is probably not far from the temperature which we strive to maintain near the skin by the regulation of our clothing. At this temperature, the rate of carbon dioxide excretion per hour per square meter was 72 cc. for T. A. and 77 cc. for F. K. In these subjects the total body surface was about 1.8 square meters, therefore the total carbon dioxide excreted per hour through the skin would be about 135 cc. (1.8 × 75) which is approximately 1.0 per cent of the carbon dioxide given off by the lungs.

Effect of changing the concentration of carbon dioxide and oxygen in the air surrounding the skin. Experiments were carried out in which the concentration of carbon dioxide in the air surrounding the skin was about 3 per cent and then at about 16 per cent. In the first instance carbon dioxide was given off, but at a slower rate than was the case when the skin was surrounded by air. In the second instance the carbon dioxide passed from the air into the skin. When the oxygen of the air in contact with the skin was reduced to about 2 per cent, then oxygen was given off by the skin; and when the oxygen concentration was increased to about 37 per cent, oxygen was absorbed more rapidly than when the skin was surrounded by air.

These experiments show that the diffusion of carbon dioxide and oxygen between the blood and the air is playing an important rôle in determining the rate at which these gases pass in and out of the skin; and since the superficial oxidation of the skin must result in the absorption of oxygen from

the air and the excretion of carbon dioxide into the air, it follows that the total gaseous exchange must be the resultant of two quite distinct processes. The relative effect of the diffusion process, as compared with the metabolic process, upon the rate at which carbon dioxide and oxygen pass through the skin cannot be evaluated until we have determined the tension of carbon dioxide and oxygen in the cutaneous blood, and thereby estimate the extent to which these gases pass through the skin in accordance with the tension difference existing between the carbon dioxide and oxygen of the blood and the air. If the diffusion process is dominant, then the rate at which carbon dioxide and oxygen will pass through the skin will depend upon the concentration of these gases in the air. To the extent that the metabolic process is dominant, the rate of carbon dioxide excretion will not be influenced by raising the carbon dioxide tension of the air to any point below that of the blood; nor should the rate of oxygen absorption be affected by raising the oxygen tension of the air to any point above that of the blood. Experiments designed to throw light upon this question are now in progress in this laboratory.

Exchange of carbon dioxide and oxygen between air and exposed tissue. Experiments were done upon cats under anesthesia to determine the approximate rate at which carbon dioxide was excreted by superficial muscle which has been stripped of its skin and exposed to air. A longitudinal median incision was made through the skin of the cat's belly and the skin reflected to expose the abdominal muscles. A brass cup, in the form of a truncated cylinder with its margin flattened to give an air-tight bearing upon the tissue, was then sealed to the muscles by means of a collodion shellac. The cup was connected to a 100-cc. mercury spirometer, and the gas throughout the system kept in circulation by means of a syringe acting as a pump. Three experiments were done and the gas for analysis withdrawn from the system at one-half hour intervals. For some reason which we do not attempt to explain, the rate of carbon dioxide excretion diminished during successive intervals. During the first half-hour interval we showed that the carbon dioxide was being excreted from exposed muscle at a rate of about 990 cc. per square meter per hour. This is about fourteen times as rapid as the rate at which carbon dioxide is given off by the skin. These experiments indicate that carbon dioxide is lost from exposed tissues at such a rate as may seriously interfere with the acid-base balance of the local circulation.

Discussion. After reviewing the previous investigations upon the elimination of carbon dioxide through the skin, we feel that the work of Barratt (7) is the only one from which reliable quantitative data may be deduced. With respect to the absorption of oxygen the available data have only qualitative value.

Though Barratt does not specify the exact area of skin from which the

carbon dioxide elimination was measured, his description leaves little doubt but that his estimations were made from a skin surface having approximately the same area as that from which our own estimations were made. Barratt carried out his experiments at two temperatures, 25°C. and 35°C. At 25°C. the carbon dioxide elimination ranged from 29.1 to 42.1 cc. per hour per square meter of skin surface, and at 35°C. ranged from 57.9 to 90.7 cc. It will be observed by reference to the carbon dioxide elimination curves in figure 4 that at 25°C. the rate of carbon dioxide excretion for T. A. is 34.5 cc. per hour, and at 35°C. the rate is 106 cc. per hour. At the lower temperatures our findings are in agreement with Barratt; at the higher temperatures, however, the rate of carbon dioxide elimination for T. A. is 17 per cent greater than the maximum value given by Barratt, and for F. K. the discrepancy is even greater.

It is of considerable significance that our observations fail to reveal a critical temperature for the carbon dioxide elimination as found by Schierbeck (3), who maintained that below 33°C. the carbon dioxide elimination is minimal and independent of the temperature, and above 33°C. the carbon dioxide elimination increases rapidly with the rise of the temperature. This conception became very generally accepted.

Next to temperature, the most important factor affecting the rate of carbon dioxide elimination is undoubtedly the variation inherent in different individuals. From inspection of the carbon dioxide elimination curves of figure 4, it will be observed that between the temperatures 28°C. and 34°C. there is a 101 per cent increase in the rate of carbon dioxide elimination for T. A., and a 216 per cent increase for F. K. Barratt found only a 95 per cent increase with a rise in temperature from 25°C. to 35°C. There is every reason to suppose that this is the characteristic response of Barratt's subject to a rise of temperature, and is still further indicative of the variations which we may expect to find in different individuals.

The nature and extent of the seasonal variation has already been referred to, but we hesitate to lay too much stress upon the influence of seasons in the absense of more extensive experimental data.

Even after the known factors affecting the rate of carbon dioxide elimination have been carefully controlled, we find that there is a diurnal variation which must, therefore, be regarded as due to a want of identity in all the conditions of the experiment, external and internal. Barratt found the maximum diurnal variation to be ± 22 per cent of the average value (the experimental error being ± 5 per cent), while we found a maximum variation of ± 19 per cent (which may be reduced to ± 17 per cent after making allowance for the experimental error). It is clear then that there are other factors, as yet undetermined, which affect the rate of carbon dioxide elimination through the skin.

It is known that the autonomic nervous system exerts a considerable regulatory effect on the blood supply of the skin. The sensitivity and nature of this regulatory mechanism varies to a marked degree in different subjects and may be responsible for these undetermined variations.

Since the respiratory quotient is always above unity, with an average value of 1.37, the excretion of carbon dioxide and absorption of oxygen cannot be due to the metabolic processes going on in the skin alone, and we must, therefore, infer that the gases are passing in and out of the blood by diffusion through the skin. To the extent that the gaseous exchange through the skin is due to diffusion, the ratio CO_2 eliminated: O_2 absorbed must depend upon *a*, the tension difference between these gases in the blood and in the air, and *b*, upon the relative rates at which the gases diffuse through the skin at a given tension difference. If carbon dioxide diffuses through living tissue at a rate which is many times greater than the rate for oxygen, as is commonly supposed, then a very slight rise in the carbon dioxide tension of the cutaneous blood would result in an increase of the ratio CO_2 eliminated: O_2 consumed which would be disproportionately great. It is probably due to this last consideration more than to changes in the metabolic rate of the skin itself that we meet with variations in the respiratory quotient of the skin ranging from 1.04 to 1.94.

SUMMARY

1. It has been shown that the rate at which carbon dioxide and oxygen pass through the skin is influenced *a*, by a change in the temperature of the air next to the skin; *b*, by individual characteristics; and *c*, by seasonal changes.

2. The conception of Schierbeck that a critical temperature exists for the carbon dioxide elimination through the skin is not confirmed.

3. The respiratory quotient of the skin varies from 1.04 to 1.94, with an average value of 1.37.

4. The cutaneous respiration under normal conditions is approximately 1 per cent of the pulmonary respiration.

5. When the carbon dioxide tension of the air is higher than that of the blood, carbon dioxide is absorbed; and when the oxygen tension is lower than that of the blood, oxygen is excreted.

6. The rate of carbon dioxide excretion from exposed muscle tissue is about fourteen times as rapid as from the skin.

BIBLIOGRAPHY

(1) Gerlach. Arch. f. Anat. u. Physiol., 1851, 431.
(2) Aubert, H. Pflüger's Arch., 1872, vi, 539.
(3) Schierbeck. Arch. f. Anat. u. Physiol., 1893, 116.

(4) LOEVY, A. In OPPENHEIMER, Handbuch der Biochemie des Menchen und der Thiere. Jena, Fisher, 2nd ed. 1923, vi, 155.
(5) STARLING, E. H. Human physiology. Philadelphia, Lea & Febiger, 4th ed. 1926, p. 986.
(6) ZUELZER, G. Zeitschr. f. klin. Med., 1904, liii, 403.
(7) BARRATT, W. Journ. Physiol., 1897, xxi, 192.
(8) DUBOIS, E. F. Basal metabolism in health and disease. Philadelphia, Lea & Febiger, 1924, p. 5.

QUANTITATIVE STUDIES OF THE NERVE IMPULSE

IV. Fatigue in Peripheral Nerve

ALEXANDER FORBES and LUCINDA H. RICE

From the Laboratories of Physiology of the Harvard Medical School

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In a recent paper Gerard and Forbes (1928) summarized evidence which has been accumulating to show that peripheral nerves are not wholly free from fatigue, as was formerly supposed. They cited the evidence of Tigerstedt (1912) that on prolonged stimulation the size of the action current declines, the evidence of Field and Brücke (1926) that the refractory phase becomes longer, and the evidence of Gerard (1927a) that the rate of oxygen consumption and heat production decreases. They then reënforced this evidence, all derived from amphibian nerves, with observations on mammalian nerves. The observations of Gerard and Forbes did not deal specifically with the size of action curent, but rather with an inability to respond to stimuli at high frequency, after prolonged activity. This is a matter of decreased excitability, that is, raised threshold, rather than decreased size of response.

Adrian[1] has raised the question whether these observations may not be the consequence of the so-called stimulation fatigue described by Gotch (1910). Gotch's observations were as follows: a frog's nerve at 5°C. was tetanized for ten or fifteen minutes, with stimuli at about 100 per second. For several minutes after cessation of tetanization, individual responses obtained by stimulation at the same pair of electrodes showed increased latency, and greatly increased duration and decreased size of the action current. When, however, the test stimuli were applied at a different pair of electrodes from those used in tetanization, there was no increased duration of response and only very slightly decreased size of response. The effect is clearly not associated with the propagated disturbance, but is confined to the region of the nerve through which the stimulating current passes. It is presumably akin to the polarization effects discussed by Bishop and Erlanger (1926) and Bishop (1927). Gotch did not state how early after cessation of tetanization he applied the first test stimulus; thus, though his observations clearly demonstrated the presence of a localized stimulation fatigue, they did not definitely rule out the presence of a func-

[1] Personal communication.

tional fatigue, associated with the propagated disturbance, which might have been of so short a duration as to disappear before his test stimuli were applied after the period of prolonged stimulation.

Such a stimulation fatigue might well account for the observations of Gerard and Forbes on decreased ability to respond to stimuli of high frequency, for the nerve was stimulated at the same point throughout the experiment, and a localized rise of threshold would obviously result in increased difficulty in exciting the nerve, even if no fatigue effects were associated with the conducted impulse. The decreased size of action current described by Tigerstedt and Gerard, and the decline in oxygen consumption and heat production are not effects confined to the site of stimulation, but are associated with the conduction of the nerve impulse. These effects, therefore, could not be explained as resulting from a localized stimulation fatigue, unless the effect of that were to raise the threshold of some of the fibers to such an extent that the stimuli which were originally maximal, i.e., able to excite all the fibers in the nerve, became submaximal, leaving some fibers unexcited. In that case the failure of some of the fibers to participate would result in all of the effects described, and would simulate the effects of fatigue, although none were present.

The prolongation of the refractory phase, on the other hand, described by Field and Brücke, is not open to such an interpretation. Bramwell and Lucas (1911) showed that the refractory phase is associated with the propagated disturbance, which is conducted throughout the extent of the nerve fiber, and not with the purely localized effect of the stimulating current. The prolongation of the refractory phase, therefore, constitutes clear evidence of a modified condition of the nerve on continued activity. So far as we are aware, there is, as yet, no clear proof that the electric response of the individual fiber actually becomes smaller in consequence of continued activity.

Method. The study of this problem has been greatly facilitated by the recent development by Campbell (1929) of a rhythmic stimulating device, depending on condenser discharges controlled by an electron tube. Two features in this device render it especially adaptable to this research; one, the perfectly regular rhythmicity of the stimuli, a condition difficult to obtain with a rotary interrupter, and the other, the fact that great changes in frequency of stimulation can be made instantly by opening or closing a switch. The changes in frequency thus produced are accompanied by little or no change in the strength or time relations of the individual stimuli.

Campbell has described several arrangements of the apparatus in which the principle of "grid leakage" is utilized in the production of intermittent oscillation. The apparatus as we have used it in these experiments is assembled according to the diagram shown in figure 1. Briefly, the prin-

ciple of its operation is as follows: the close-coupled inductances in the grid and plate circuits cause radio-frequency oscillations in the electron tube; because of the high resistance in the "grid leak" a negative charge accumulates on the grid and causes the oscillations to cease; the grid condenser is then discharged through the leak until the potential of the grid rises enough to permit the oscillations to begin again. In this way intermittent periods of oscillation result, and the duration of the complete cycle of changes depends on the resistance of the grid leak and the capacity of the grid condenser, an increase in either of these quantities increasing the duration of the cycle. Rhythmically repeated shocks are led off from

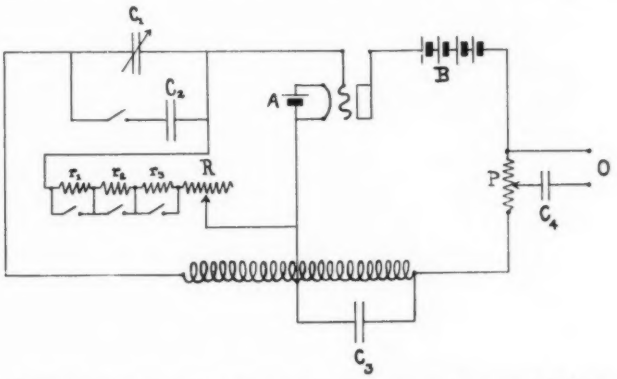

Fig. 1. Diagram of stimulating apparatus. Electron tube, type UX201A.

A, filament battery, 4.5 volts. *B*, plate battery, 90 volts. C_1, variable condenser in grid circuit. C_2, fixed condenser in grid circuit, 0.0025 mf. C_3, fixed condenser in oscillating circuit, 0.0025 mf. C_4, series condenser in output, 0.0025 mf. *R*, variable resistance in grid leak, 0 to 8 megohms. r_1, r_2, r_3, fixed resistances in grid leak, 2, 5 and 10 megohms respectively. *P*, potentiometer for varying strength of stimuli. *O*, output leads connected directly with stimulating electrodes.

the plate circuit through the series condenser, C_4, to the nerve, and their intensity is varied at will by varying the E.M.F. tapped off from the potentiometer, *P*. Changes in the potentiometer setting cause small changes in the frequency of discharge, the frequency decreasing slightly as the voltage is increased.

The wave form of the current led from this apparatus to the nerve was studied by connecting the output wires directly with the string galvanometer, protected by a suitable shunt, and observing the result with the revolving mirrors (Forbes and Mann, 1924); also by photographic recording on a moving film (fig. 2). Although the wave form is not quite as simple as in some of Campbell's records, the result is satisfactory for nerve stimulation, for with the maximum stimulus of which the apparatus is capable

we have never seen the slightest evidence of compound (repetitive) stimulation from a single shock, as is sometimes found in the case of induction shocks from a coil with an iron core. Evidently the secondary waves occur so early in the nerve's refractory phase following the response to the main wave that they never evoke a second response.

By measuring the excursions on the photographic film we found that the current in the output was approximately proportional to the reading on the potentiometer scale, provided the other values were kept constant. In stimulating the cat's peroneal nerve with this apparatus, we have usually found the threshold at potentiometer readings between 1 and 2 in a scale of 10. From this it appears that the range in which the threshold usually fell lay between 10 and 20 per cent of the maximum obtainable stimulus.[2]

The perfect regularity of the rhythm and the uniformity of the successive stimuli are shown in figure 2, in which are reproduced galvanometer records of the current in the output at several different frequencies of discharge. It will be seen that the wave form and size of excursion, as recorded in the galvanometer, remain the same for the individual stimuli, irrespective of frequency, until this is increased to the point at which the galvanometer excursions overlap. Since the lag of the galvanometer is appreciable, it is impossible to tell from these records whether at higher frequencies there

[2] The strength of the stimuli may be increased to a higher range of values by increasing the capacity of the output condenser, C_4, or by use of a tube with different characteristics.

Fig. 2. Rhythmic currents from stimulating device, recorded directly with string galvanometer.

	GRID CONDENSER	GRID LEAK	POTENTIOMETER	GALVANOMETER SHUNT
	microfarads	*megohms*		*ohms*
A	0.0025	18	7	10,000
B	0.0025	18	3	10,000
C	0.0025	1.5	8	1,000
D	0.0025	1.0	8	1,000
E	0.0025	1.7	8	1,000
F	0.0025	1.0	8	1,000
G	0.0025	1.5	10	1,000
H	0.0025	1.0	10	1,000
I	0.00048	3.5	10	1,000

Magnification, 470; string tension 244 m. per amp. (see Forbes and Ray, 1923, THIS JOURNAL, lxiv, 435). Time marker shows 0.01 second intervals. Records E-I, inclusive, were made on the same day, and the speed of film was the same in all. In A the major portion of the initial excursion (downward) is so rapid as to be invisible in the record.

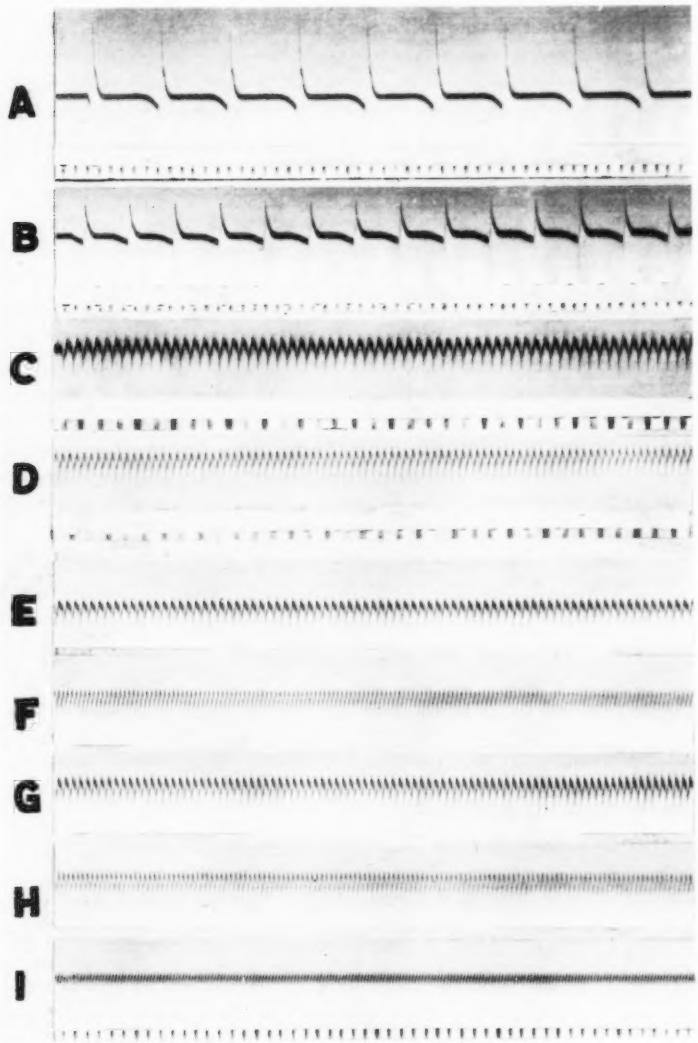

Fig. 2

is a change of wave form or not. Observations on nerves make it clear that the stimuli are effective at all frequencies up to the limit of the nerve's ability to follow them with separate responses.

It is customary in using break induction shocks to make the kathode distal, i.e., nearest to the leads, in order to avoid the possibility of anelectrotonic block. In conformity with this practice we connected the apparatus with the nerve so that the electrode nearest the galvanometer leads should be the kathode during the initial phase of the shock. In a few tests we reversed the wires and found but little difference in threshold, and no evidence of electrotonic block, even with the strongest stimuli; i.e., the action currents appeared the same whichever way the wires were connected.

In order to minimize changes in frequency due to electrostatic effects the apparatus was installed in a box lined with a shield of copper wire netting, and the shield was grounded.

To initiate a series of stimuli three methods were tried. The first was to have the output already connected with the electrodes on the nerve, and start the series of discharges by closing the switch in the filament circuit, which causes the filament to glow and thus initiates the rhythmic series of stimuli. The disadvantage of this method is that the filament does not instantly reach its operating temperature; consequently while it is warming up, the stimuli are delivered to the nerve with changing frequency. The second method was to have the filament circuit closed and the oscillating electron tube already in operation, then to commence stimulation by closing a double-pole switch connecting the output with the stimulating electrodes on the nerve. The disadvantage of this method was that closure of the switch resulted in a transient current through the galvanometer, rapidly deflecting the string just before the beginning of the first action current, and consequently reducing the resulting excursions.

The third method, which was finally adopted, was to have the oscillating circuit already in operation and the output connected with the nerve, and to commence stimulation by opening a short-circuiting switch in the output circuit. When this method is used small electric artefacts appear in the record while the short-circuiting switch is closed (see fig. 3). In order to determine whether these excursions were caused by submaximal action currents due to stimulation by the surge of current into the nerve in spite of the short-circuit, the nerve used in one experiment was killed by soaking in ether and then replaced on the electrodes in the moist chamber, and the stimulating current was applied as before. Thereupon small excursions appeared in the record, about the same size as those appearing before the beginning of stimulation in figure 3 (i.e., before the short-circuit was open). It is interesting to note that, in the case of the dead nerve, when the short-circuit was opened the excursions became slightly smaller and more

frequent. In this particular experiment, before the nerve was killed the excursions preceding the opening of the short-circuit were slightly bigger than the corresponding excursions after the nerve was killed. From this it appears probable that in this experiment even the short-circuited shocks sufficed to stimulate a few fibers and thus produced a small action current. In most experiments probably very few fibers, if any, were stimulated by short-circuited shocks, judging from the fact that the preliminary excursions were of the same size as those which depended on artefacts in the case of the dead nerve under very nearly the same electrical conditions. In general the artefact was so small that it added little to the total excursion in the case of a full-sized action current, but in the case of high-frequency stimulation, in which the action currents were very small, the artefact may have contributed a considerable share of the observed excursions.[3]

In these experiments we used the peroneal nerve of the cat. The nerves were carefully dissected from hip to knee under ether anesthesia by Dr. H. Davis, who was engaged in another research which demanded the removal of this nerve from the field of operation. The nerve, immediately after removal from the animal, was placed in Ringer solution at a temperature between 20° and 25°C. After a few minutes it was set up in a moist chamber with Ag-AgCl electrodes about 15 mm. apart for leading off the action currents. The nerve was crushed at a point 2 or 3 mm. proximal to the distal lead, to render the responses monophasic. The temperature of the moist chamber during the observations was about 25°C.

The action currents were recorded with a Hindle string galvanometer with 1.5 mm. air gap. Since the last reported experiments with this instrument, it has been installed in a new location on the ground floor, where it is mounted on a slab of reinforced concrete, resting on a felt pad supported by a reinforced concrete foundation built from the basement through a hole in the floor. The concrete foundation makes no contact with the floor, the space between being loosely filled with thick felt.

The recording camera has been described elsewhere (Forbes and Thacher, 1920) it is mounted on a table, which also carries a metal track on which the revolving mirrors can at a moment's notice be slid in front of the camera for the purpose of viewing the responses which are to be recorded. In these experiments the mirrors were used to determine the threshold and maximal values of the stimuli, and thereafter records were made with the camera.

The galvanometer string used in all these experiments was of gilded quartz, 2.0μ in diameter and of 25,000 ohms resistance. The magnification was varied from 410 to 500 by means of an adjustable ocular.

[3] In a subsequent test it has been found that removal of the ground connection from either the shield around the stimulating apparatus or the galvanometer string, greatly reduces the artefact.

Other details of procedure will be described as the need arises.

Results. We shall first describe the simple procedure of stimulating the nerve, after a period of rest, with a series of stimuli at high frequency, and recording the resulting action currents. In selecting the strength of stimuli, it is important to make them supramaximal, for the more they exceed the maximal value at the outset, the less likely are they to become submaximal in consequence of localized stimulation fatigue, and thus cause a deceptive failure of some fibers to participate in the response. Unfortunately the maximal value of the stimulus is not always very clearly defined. Sometimes, as the strength of stimulus increases, the size of the monophasic action current reaches a definite maximal value when the stimulus is about four or five times the threshold strength, and thereafter remains constant over a wide range (cf. Forbes and Gregg, 1915, fig. 1; Adrian and Forbes, 1922, figs. 11, 13 A and 14 A). Sometimes, as the

TABLE 1

Correlation of action currents with strength of stimulus

DATE	POTENTIOMETER READING					
	3	4	5	6	8	10
	mm. galvanometric excursion					
January 10			5.2	5.3	5.6	5.7
February 14		7.7	7.8		7.9	
March 12		3.6	3.6	3.7	3.7	4.0
March 21	6.9	7.4	7.9	7.9	8.0	8.2
May 20	7.2	8.6	9.0	9.1	9.1	

strength of stimulus is increased, the action current will continue to increase slightly even after an approximately maximal value has been reached, until the result is confused by electric artefact or repetitive stimulation (cf. Adrian and Forbes, 1922, figs. 13 B and 14 B). This effect may be partly due to abnormally high threshold in some of the fibers in consequence of slight damage caused by handling, and it may be in part due to the normally high threshold which Erlanger, Bishop and Gasser (1926) have found to characterize some of the constituent fibers in a mixed nerve. In the present experiments there was usually a very slight increase in the size of the action currents with increasing strength of stimuli, through the upper half of the potentiometer range. This continued increase is probably due in part to the increasing augmentation of the excursion by the electric artefact. Table 1 shows the correlation of action current with strength of stimulus from a value well above threshold to the maximum obtainable from the apparatus, in the case of four of the

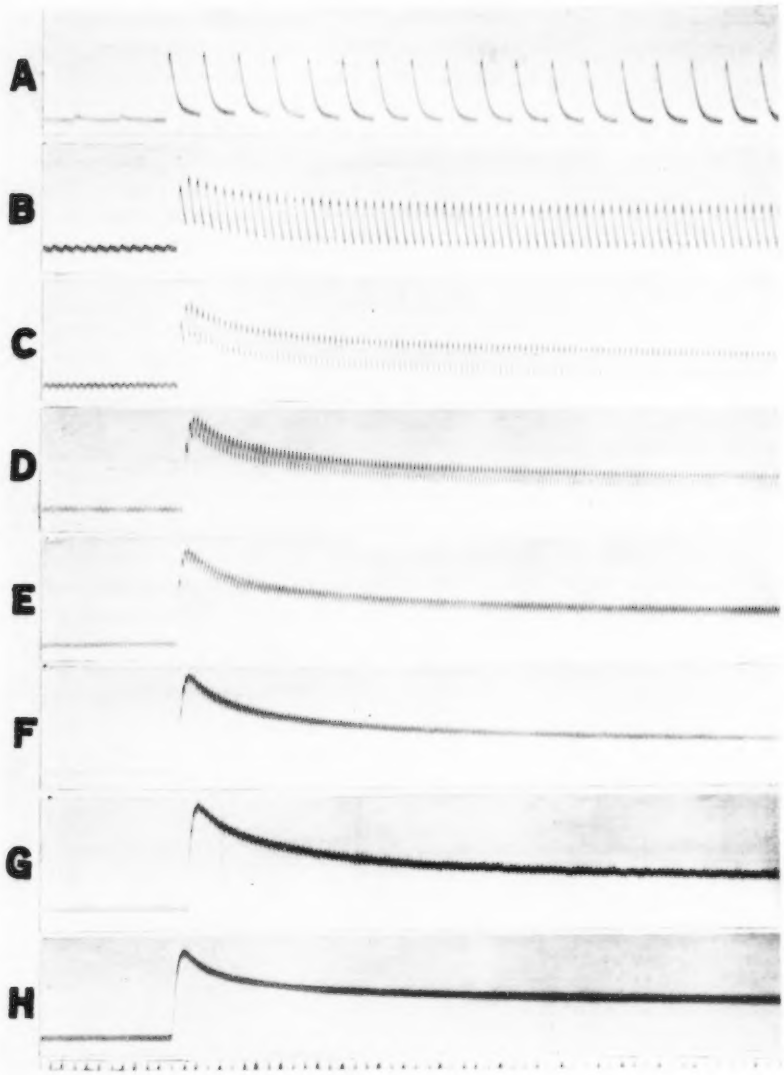

Fig. 3. Action currents of peroneal nerve of cat. Beginning of rhythmic stimulation at various frequencies. March 26, 1929, A, 35; B, 138; C, 218; D, 306; E, 360; F, 410; G, 515 stimuli per sec. H, direct current passed through twine soaked in Ringer in series with the same electrodes, to show polarization, April 15, 1929. Magnification, 470; string tension 244 m. per amp. Speed of film the same in all, shown by time marker (0.01 sec.) at bottom.

seven nerves used in these experiments. These observations were made with a frequency of stimulation so low that each response occurred some time after the relative refractory period following its predecessor, and was therefore full-sized.

Most of our observations were made with the potentiometer set at 8 (the maximum setting was 10), which meant that virtually all the fibers in the nerve participated in the responses, and a considerable rise in threshold would occur before any appreciable number of them would fail to respond. By repeating the observations with the potentiometer at its maximum, it was possible to control the question of failure of the stimuli to remain maximal because of a rise of threshold.

The simplest procedure employed in testing for fatigue was to stimulate a rested nerve with a prolonged series of stimuli at high frequency, maintaining the frequency constant throughout the series. The results of several such tests at frequencies of stimulation from 138 to 515 per second are shown in figure 3, together with one record in which the frequency of stimulation was so low that the nerve was completely recovered from its refractory period after each response, and the action currents were therefore all full-sized. It will be seen that with 138 stimuli per second the impulses following the first occur comparatively late in the relative refractory period, and are therefore nearly full-sized. With the higher frequencies the impulses are increasingly subnormal as the frequency is increased, and the successive responses are evoked increasingly early in the relative refractory period. With the highest frequencies, the series begin with an obviously summated excursion of the string. This does not signify a summated electric response, since the inertia of the string is such as to cause a mechanical accumulation of displacement. After the point of maximum total excursion, the mean deflection of the string declined fairly rapidly to a new base line. This decline might be due to the stimulation fatigue of Gotch, to a true functional fatigue associated with the propagated disturbance, or to electrode polarization. The silver chloride electrodes used are not completely non-polarizable, and the effect of sustained current on the base line, due to their polarization, requires investigation. Therefore a small E.M.F. was tapped off from a battery circuit and introduced into the circuit of the string galvanometer, connected in series with a wick of twine through the Ag-AgCl electrodes used for leading off the action currents of the nerve. A photographic record was made while the battery circuit was closed and the E.M.F. was thus introduced into the circuit. A typical example of such a record is included in figure 3. It was made with the electrodes untouched for three weeks after their use in the experiment in which we made the nerve records shown in this figure. Adding more chloride to these electrodes, caused an increased resistance and a decrease in the polarization effect (i.e., the relative over-

shoot beyond the steady deflection). From this experiment it is clear that most of the decline in the base line appearing in the record of action currents at high frequency (e.g., fig. 3) was due to electrode polarization. For evidence of decline in the size of the action currents we must look for decrease in the amplitude of the individual excursions of the string. Whenever the frequency of stimulation is high enough to place the second response in the relative refractory phase following the first, the size of the second response will, of course, be subnormal. The refractory phase, although in a certain sense a fatigue phenomenon, must be clearly distinguished from the equilibration type of fatigue, which is the subject of our experiments. This distinction has been discussed by Gerard (1927b). In the analysis of string galvanometer records the effects of inertia of the string and of tension due to displacement are so complex that we can only fairly compare successive excursions after an approximately steady state has been reached, that is, when at the beginning of each response the displacement and rate of motion of the string, in consequence of the previous response, are approximately the same. All of our records show a gradual decline in the size of the individual deflections, which is appreciable during the first quarter of a second. This decline continues, growing more and more gradual, but still being appreciable for many seconds. After the first second the rate of decline is so slow that it is difficult to observe it in a continuous record. If, however, the film is stopped for ten or fifteen seconds, while stimulation is continued, the excursions when recording is resumed show a clearly visible further decline (see fig. 5).

The decline in the size of the action currents shown in our records, on continued stimulation at high frequency, might be due, as Adrian has suggested, to localized stimulation fatigue. This could only be so, however, if the result of such stimulation fatigue were to render some fibers inexcitable to stimuli of the strength employed, or if the local effect caused a slowing of conduction sufficient to increase the temporal dispersion of the impulses in the individual fibers enough to reduce the galvanometric excursion (see Davis and Brunswick, 1926); otherwise, an effect localized in the vicinity of the stimulating electrodes could not affect the response recorded at the leads several centimeters away on the nerve, for it is well established that the electric response, if not extinguished in traversing an impaired portion of the nerve fiber, regains its normal size promptly on emerging into an uninjured portion of the nerve (Adrian, 1912; Kato, 1924; Davis, Forbes, Brunswick and Hopkins, 1926).

In order to control the possibility that the observed decline in galvanometric excursions might be due to localized stimulation fatigue, we arranged two pairs of stimulating electrodes in the moist chamber, with a double-pole rocking key whereby the stimuli could be rapidly shifted from one pair of electrodes to the other (fig. 4). In practice we found that when

the shift was made, stimulation was interrupted for only about one hundredth of a second. In this way stimulation fatigue was eliminated from that part of the nerve to which the stimuli were applied in the comparative tests. The procedure was as follows: stimulation was begun at the distal pair of electrodes (nearest the galvanometer leads); after about a second of stimulation at high frequency the stimuli were shifted to the proximal electrodes. After one or two more seconds of stimulation the film was stopped, but stimulation was continued through the proximal electrodes for 15, 20 or 25 seconds. Just before the end of this period the film was started again, and while the record was being made the stimuli were quickly shifted back to the distal electrodes. Stimulation was continued there for a second or so and then stopped. In some experiments, as an additional control, the rested nerve was stimulated for a second or so through the proximal electrodes alone.

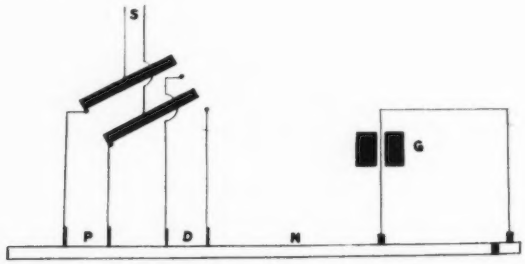

Fig. 4. Diagram of arrangement for shifting the stimuli from one pair of electrodes to another.

S, wires leading from stimulating apparatus; *P*, proximal; *D*, distal stimulating electrodes; *N*, nerve; *G*, string galvanometer.

Typical observations of this sort are shown in figure 5. The shift of the stimuli from distal to proximal electrodes usually resulted in an immediate slight decrease in the size of the excursion. Continued stimulation resulted in a further gradual decline in the size of response, such as has already been described. Shifting back to the distal electrodes caused a slight increase in the size of the excursions. Immediately after this second shift the excursions were about as large as they were just before the first shift (distal to proximal), but they never returned to their initial size.

The following factors may have contributed to the immediate decrease in the size of excursions following the shift from the distal to the proximal electrodes. 1. Conduction over a greater distance would cause increased dispersion, i.e., loss of synchronism in the responses of the individual fibers. 2. Higher threshold at the proximal electrodes might render the stimuli submaximal (i.e., ineffective for some fibers). Davis and Bruns-

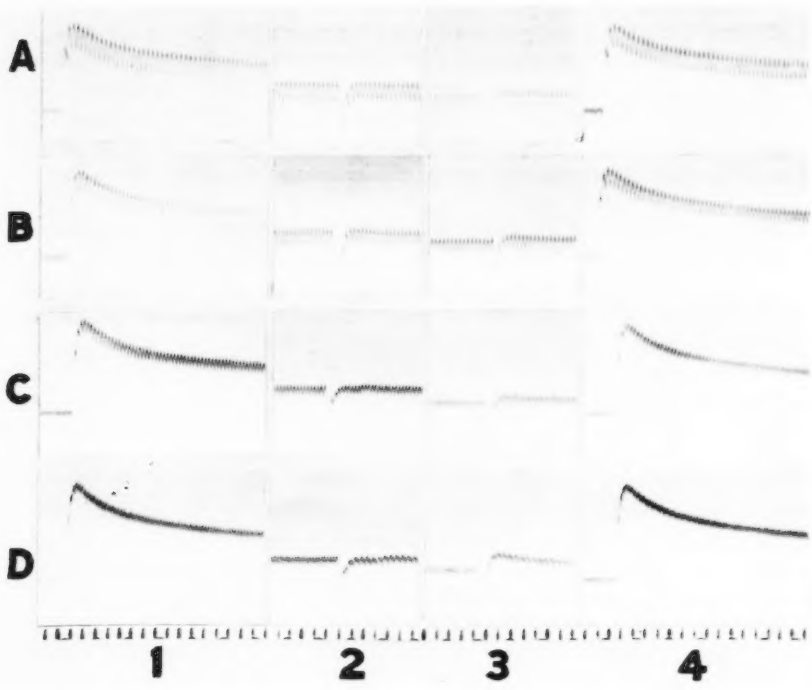

Fig. 5. Action current records showing the effect of changing the point of stimulation.

1, beginning of stimulation; *2*, shift from distal to proximal electrodes; *3*, return to distal electrodes after prolonged stimulation; *4*, stimulation of rested nerve through proximal electrodes. Records all made from the same nerve within 20 minutes.

	FREQUENCY	INTERVAL BEGINNING OF STIMULATION TO FIRST SHIFT	FIRST SHIFT TO SECOND SHIFT
		seconds	*seconds*
A	254	0.67	10
B	300	0.93	15
C	356	1.02	15
D	410	1.34	15

Time recorded in 0.01 second intervals below.
Speed of film constant throughout experiment.
String tension and magnification as in figure 3.

wick (1926) showed a decrease of functional capacity extending as far as 20 mm. from a crushed point on a similar nerve trunk. In our experiments the proximal stimulating electrodes were applied within less than 20 mm. of the point at which the nerve was crushed by the ligature which supported the proximal end. 3. Decreased electric artefacts, due to the greater distance of the stimulating electrodes from the lead, might cause a slight decrease in the excursions. 4. The establishment of an electrotonic block due to the localized effect of the stimulating current in the vicinity of the distal electrodes, now interposed in the path between the proximal electrodes and the leads, might produce a similar effect.

If electrotonic block were the principal cause of the decrease immediately following the shift from distal to proximal electrodes, there should be a fairly rapid return of the excursions to the size existing just before the shift was made. Instead of this there is a continued progressive decline in the size of the excursions. Electrotonic block, therefore, can not contribute appreciably to the observed abrupt decrease. The other three factors all may be contributing causes; we are unable to determine which of the three is the most important. When the frequency of the stimuli is very near the upper limit of the nerve's ability to respond to every stimulus, the shift from distal to proximal electrodes is followed by an apparently complete alternation of response, i.e., the nerve, instead of responding to every stimulus, responds only to every other stimulus (cf. Beritoff, 1913). This is shown in figure 5 D. The inference from this observation is that at the proximal electrodes the functional capacity of the nerve is somewhat impaired due to proximity of the ligature, and the failure of all the fibers to respond to every stimulus results either from a considerably raised threshold, or a prolonged refractory phase, or from a combination of these two causes.

From the point of view of our present problem the important feature in such observations as those shown in figure 5, is the fact that when, after the nerve had been stimulated through the proximal electrodes for from 10 to 20 seconds, the stimuli were shifted back to the distal electrodes, the galvanometric excursions never returned to the same size that was found in the first few responses after the string had reached its maximum displacement. The conclusion to be drawn from this is that a slight degree of fatigue had been established, quite independent of the localized stimulation fatigue.

It is possible that the decrease in the size of galvanometric excursions upon which this conclusion rests is due solely to a prolongation of the refractory phase. That such a prolongation occurs is established by the work of Field and Brücke. At the high frequencies of stimulation under consideration, each impulse after the first is set up during the relative refractory period following its predecessor, and is therefore subnormal.

As the refractory period becomes prolonged these successive impulses, set up with a constant frequency, will fall relatively earlier in the recovery period, and will thus be smaller in size. Thus the present observations, while clearly showing a decreased size of response, independent of any localized stimulation fatigue, may nevertheless be nothing more than a confirmation of the observations of Field and Brücke on the increased duration of the refractory period resulting from prolonged stimulation.

It is desirable to determine whether impulses occurring when a nerve has completely recovered from its refractory period are also reduced in size in consequence of a prolonged period of functional activity. To test this question we made use of the provision in the stimulating apparatus for instantaneous changes in the frequency of stimulation. The nerve was first stimulated at a low frequency (less than 40 per second), then fatigued with stimulation at a high frequency (between 200 and 600 per second) for 15 to 25 seconds, then tested with stimuli of the same low frequency as at the beginning.

In the first experiment the stimuli were all applied at one pair of electrodes. In this experiment also a double shift of frequency was made by cutting out large units of both resistance and capacity in the grid circuit, thus the size of the action current, before and after prolonged stimulation, was tested with stimuli at 19 per second and the fatigue effect was produced by stimulation for 20 seconds at 317 per second. The galvanometric excursion immediately after the return to the slowest frequency of stimulation, after the period of prolonged high frequency, were reduced to about 83 per cent of their initial size. On continuation of the slow frequency stimulation the size of the action current gradually returned to an equilibration value slightly below that obtaining before the high frequency stimulation, equilibrium being reached in about 0.75 second. This observation appears to show a definite reduction in the size of response through fatigue.

The possibility remains that this reduction may have been due to stimulation fatigue. As already pointed out this could only be the case if conduction were greatly retarded and temporal dispersion resulted, or if the threshold of a considerable number of the fibers were raised to such a point that the stimuli which previously excited them now failed to do so, for the responses were led off so far from the stimulated region that the individual axon potentials must have returned to normal before reaching the leads. Two methods were used for controlling these possibilities; one was to increase the strength of stimuli while the responses were still reduced in size, and see whether the galvanometric excursions immediately increased to their initial value in consequence; the other was to employ the method already described of applying the stimuli to two different pairs of electrodes. In the remaining six experiments both methods of control

were used. In one of these the double frequency shift was used (i.e., by changing both resistance and capacity). In this case there was very little reduction of the action current, following prolonged high frequency stimulation. The reason for this probably lay in the fact that during the transition stage, when the stimuli were of intermediate frequency, there was a large measure of recovery from the fatigue caused by the highest frequency of stimulation. In the subsequent experiments the capacity of the grid condenser was maintained at a large value and the change of frequency made only by short-circuiting the 10-megohm resistance in the grid leak; thus the change from the highest to the lowest frequency was made instantaneously. With this arrangement the frequency of stimulation could be instantly shifted from about 30 per second to over 500 per second. The higher frequency was regulated by adjustment of the variable resistance. Frequencies between 200 and 500 per second were used in our observations of this type. In every case in which this procedure was employed there was an appreciable reduction in the excursions immediately following the cessation of high frequency stimulation.

The effect of increasing the strength of the stimuli, while the reduced size of action current persisted, is illustrated by the following typical experiment. Stimulation was begun with the potentiometer set at 8 (i.e., 80 per cent of the maximum stimulus) and with 37 stimuli per second, applied at the proximal electrodes (no electrode shift was made in this observation). After 1.5 seconds the frequency was changed to 250 per second. This was continued for 20 seconds; then the frequency was changed back to 37 per second. At 1.04 second after the change of frequency, the stimuli were increased in strength by raising the potentiometer from 8 to 10. This change was completed 1.21 seconds after the change of frequency. (The time occupied in this change was revealed on the film by the recorded slight change of frequency, which was incidental to the change of strength.) The size of excursions plotted against time is shown in figure 6. It appears that during the increase in the strength of stimuli there was only very little more rapid increase in the size of response than that which was occurring progressively with the lapse of time after high frequency stimulation. Inasmuch as there was, correlated with similar increases in the strength of stimuli in all experiments, a slight increase in the size of excursion, irrespective of recovery from fatigue, the slight additional increase observed in this experiment probably does not signify that the reduced size of response after high frequency stimulation was due to submaximal stimulation resulting from a localized effect. Other similar observations were made in this and other experiments; in some of these the strength of stimuli was increased sooner after the change of frequency, so that its effect, if any, could be noted earlier in the recovery of the responses from their reduced size. In all cases the result was essentially the same as the example shown in figure 6.

There still remains the possibility that the reduced size of response immediately following the prolonged period of high frequency stimulation was due to increased dispersion in time of the action potentials in the individual fibers, caused by the localized effect at the stimulating electrodes. The observations of Gotch, in which he demonstrated the existence of a localized stimulation fatigue, clearly showed that a prominent feature of the effect was slow conduction in the vicinity of the stimulating electrodes. This fact was shown both by the increased latency of response at remote electrodes and by the increased duration of the action current at that point. Since the leading off electrodes were shown by his control experiment to be far enough away from the stimulating electrodes to be outside of the region modified by the stimulating current, the time relations, as well as

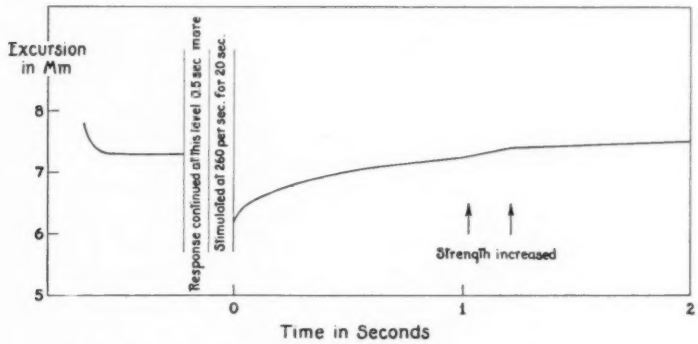

Fig. 6. Galvanometric excursions plotted against time, showing initial decline on stimulation at 37 per sec., further decline after prolonged stimulation at 250 per sec., recovery in successive responses on return to 37 per sec., and slight increase in excursions on increasing strength of stimuli.

the magnitude of the electric response in the individual fiber, must have been normal, that is, just the same as if the impulse had not been initiated in a modified region; for it is well established that the size and time relations of the response depend only on the local condition of the fibers and not upon that of other portions of the nerve which it has traversed. In our experiments, if the dispersion of the individual impulses in consequence of localized stimulation fatigue was great enough, this effect might be sufficient cause for the observed reduction in the size of response after prolonged stimulation.

The only positive control for this possible explanation was to make the test for size of response through the distal stimulating electrodes and to apply the fatiguing stimuli through the proximal electrodes. If the reduced size of response after prolonged stimulation were traceable to the

results of stimulation fatigue, we should find, following the shift to the distal electrodes, an immediate return of the size of response to its initial value. This was never observed. Some of our observations on this point might be criticized on the ground that the shift from proximal to distal electrodes was not made until a second or more after the change from high to low frequency of stimulation, and by this time the size of response had already almost returned to its initial value. To meet this objection a large number of observations were made in which the shift of electrodes was made within a quarter of a second of the change of frequency and therefore when the recovery of the size of response was still incomplete. The shift of electrodes was so rapid that in low frequency tests it usually fell between stimuli, and the record gave no indication when it occurred. In one experiment a signal magnet was arranged to throw a shadow on the film and record the moment of shift from the proximal to the distal electrodes. Records of the action current before and after high frequency stimulation in several experiments are reproduced in figure 7, and typical curves plotted from measurements of the excursions are shown in figure 8. From these observations it will be seen that in no case when the shift of electrodes was made while the action currents were still reduced was there a sudden return to the initial size. In some cases there was a slight increase correlated with the shift, but this increase was no greater than the difference between the responses obtained from the two pairs of electrodes in the rested nerve. The evidence, therefore, appears to be conclusive in demonstrating the presence of a real decrease in size of response in the nerve trunk, associated with the propagated disturbance and independent of the stimulation fatigue described by Gotch.

An attempt was made to gauge the percentage decline in the size of response after prolonged stimulation in these experiments. All of the nerves tested showed a slight decline in the size of all responses after the first, even at the low frequencies of stimulation (34–37 per second). On measuring the first excursions after the shift from high to low frequency of stimulation, we found, in nearly all experiments, a decline to between 70 and 80 per cent of the excursion in the rested nerve.[4] The decline appeared to be slightly greater when the duration of the high frequency stimulation was increased from 15 to 20 or 25 seconds, but the difference was so small that the major effect could clearly be ascribed to the first 15 seconds. The

[4] In those experiments in which stimulation was shifted from distal to proximal electrodes, and back again, the initial size of response only appeared on stimulation at the distal electrodes. Since the test stimuli immediately after the return to low frequency were applied to the proximal electrodes, it was necessary to estimate what would have been the initial size at the proximal electrodes by a correction factor based on a previous comparison of the responses of the rested nerve on stimulation at the two pairs of electrodes. This correction amounted to about 3 per cent.

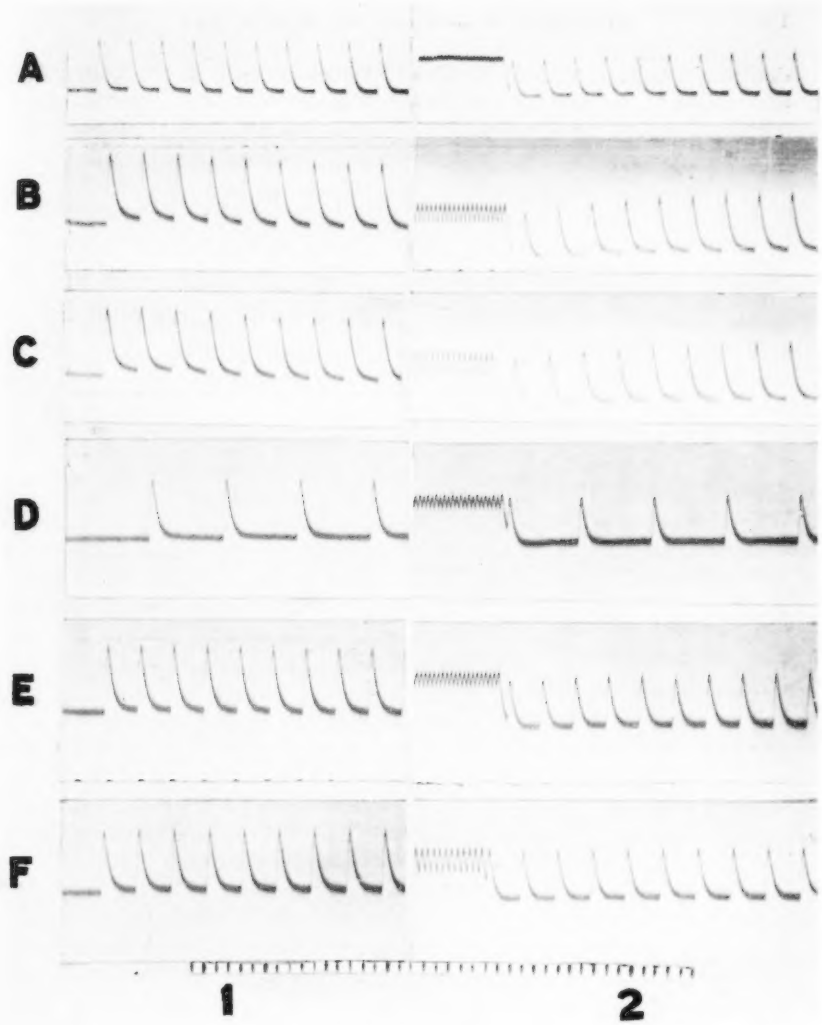

Fig. 7. Records of six observations from three experiments, showing the effect of high frequency stimulation on subsequent responses at low frequency.

1, beginning of low frequency stimulation after rest; distal electrodes. *2*, end of high frequency stimulation and return to low frequency; proximal electrodes.

A, Feb. 14, 1929, high frequency 316 per sec. for 25 sec. B and C, March 26, 1929, high frequency for 15 sec.; B, 254 per sec.; C, 215 per sec. D, E and F, May 20, 1929, high frequency for 25 sec.; D, 294 per sec.; E, 284 per sec.; F, 200 per sec.

Speed of film (approximately the same in all) shown by time marked below (0.01 sec.).

String tension and magnification as in figures 3 and 5.

decline seemed also to increase slightly with increase in the frequency of stimulation up to a maximum in the neighborhood of 300 per second.

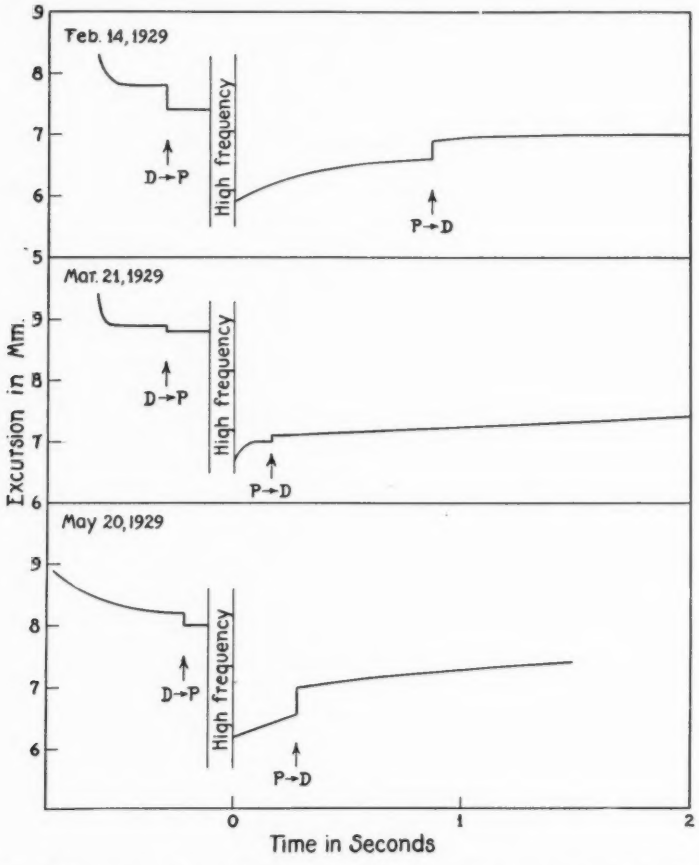

Fig. 8. Excursions plotted against time in three experiments showing effect of high frequency stimulation controlled by electrode shift. Time of shift from distal to proximal electrodes shown by "*D–P*"; from proximal to distal "*P-D*".

Feb. 14, low frequency 37 per sec.; high frequency 316 per sec. for 25 sec. (same observation as in fig. 7 A). March 21, low frequency 36 per sec.; high frequency 250 per sec. for 15 sec. May 20, low frequency 16 per sec.; high frequency 294 per sec. for 25 sec. (same observation as in fig. 7 D).

With higher frequencies the decline seemed to become slightly less,—an effect possibly depending on the failure of alternate stimuli to excite all

the fibers. But the effect of varying the frequency was so slight and the correlation with the amount of decline so uncertain that it would be impossible to make a positive statement on this point without many more observations.

The first three or four responses after the frequency was changed from high to low, showed rapid recovery, the excursions returning nearly to their previous equilibrium value. Thereafter there was a more gradual return for about one second, after which no further increase could be measured (fig. 6). The new equilibrium value was usually about 5 per cent less than that obtained before high frequency stimulation, but our observations were rarely continued more than 3 or 4 seconds after returning to low frequency. Had we prolonged the low frequency stimulation sufficiently, both before and after the high frequency, it is quite possible that the equilibrium values would have proved identical.

One nerve, in which the maximal excursions were only about half the usual size, showed a much smaller relative decline than the others. In this nerve the first response after cessation of high frequency stimulation was regularly from 86 to 88 per cent of the initial size. Since this experiment was unique in the series, we cannot say whether the small decline was related to the small size of initial response.

We have already mentioned the prolongation of the refractory period described by Field and Brücke. The question arises whether the prolongation in our experiments was sufficient to place the successive responses in the relative refractory periods following their predecessors, even at the low frequency of stimulation (34–37 per sec.). If this were the case the observed reduction might be simply the well known subnormality of response associated with the relative refractory phase. The total time of recovery (i.e., absolute and relative refractory periods together) in a rested mammalian motor nerve at 25°C. is about 5 or 6 σ. Field and Brücke found, on stimulation for several minutes, a 4-fold to 9-fold increase in the refractory period, as measured by their method (which probably determined approximately the absolute refractory period). If the relative refractory period is correspondingly prolonged, we might find the impulses subnormal for 40 or 50 σ after a previous impulse, provided the same degree of fatigue were attained in our experiments as in theirs. The interval between stimuli at slow frequency in our experiments varied from 27 to 30 σ; therefore it is possible that after the periods of prolonged high frequency stimulation the successive stimuli were within the relative refractory period. On the other hand, the prolongation of the refractory period was probably a good deal less in our experiments, with only 20 seconds of fatiguing stimuli, than in those of Field and Brücke, with stimulation for 10 minutes. This consideration, together with the very gradual return to normal during the last part of the relative refractory period, makes it doubtful whether so

large a decline as the observed 20 to 30 per cent could be due to prolonged relative refractory period.

Another feature of the relative refractory period, besides subnormal response, is slowed conduction (Forbes, Ray and Griffith, 1923; Gasser and Erlanger, 1925). Since the refractory period differs in different fibers, we should expect differential slowing in the case of an impulse set up in the relative refractory period. This fact would provide the condition for temporal dispersion, which, by reducing the galvanometric excursion, might prove to be a source of confusion. The observations of Davis and Brunswick (1926) indicated that the dispersion must amount to about 4 σ before the action currents will be far enough out of phase to reduce appreciably the excursions with a galvanometric string of 2.75 μ diameter. Probably a dispersion of 2 σ would be necessary in the case of the smaller string used in our experiments. With the proximal lead only 3 cm. from the point of stimulation the conduction time for the most rapid fibers would be less than 1 σ. Therefore it is improbable that a significant number of impulses would be retarded by an additional 2 σ in consequence of

TABLE 2

Action currents; as percentage of initial response of rested nerve

LOW FREQUENCY OF STIMULATION	EQUILIBRATION VALUE BEFORE HIGH FREQUENCY	FIRST RESPONSE AT LOW FREQUENCY AFTER HIGH FREQUENCY
16 per sec.	97	79
35 per sec.	94	75

the very slightly reduced velocity of conduction occurring toward the end of the relative refractory period.

Although these possible sources of confusion due to the relative refractory period are improbable in our experiments, the question was tested in a final experiment. A large number of observations were made, similar to those already described, but with the test stimuli applied at only 16 per second. For comparison we repeated the procedure with the usual frequency of 35 per second. In order to make the rapid shift from high frequency (200 to 300 per sec.) to the low frequency of 16 per second, the switches short-circuiting the three fixed resistances in the grid leak were opened simultaneously.

With the frequency of the test stimuli as low as 16 per second, we may be reasonably certain that, even during fatigue, each successive impulse is well outside of the relative refractory period following its predecessor. If in the majority of our experiments in which the test stimuli were applied at 35 per second the observed decline was due to prolonged relative refractory period, this decline should disappear when the stimuli are spaced out to 16 per second. In this final experiment the action currents were

the largest obtained in any of our series, and records favorable for a fairly accurate comparison were therefore obtainable. In most of the observations the high frequency stimulation was continued for 25 seconds. When the subsequent test stimuli were applied at 16 per second the responses showed approximately the same decline as those in most of our previous experiments, namely, to between 70 and 80 per cent of the value found in the rested nerve. In table 2 is shown the average percentage of the initial size, to which the responses declined both before and immediately after high frequency stimulation, in 9 cases in which the test stimuli were at 15 to 16 per second, and in 2 cases with 36 per second for comparison. In every case included in this average the high frequency stimulation was continued for 25 seconds, and the stimuli were applied at the same electrodes throughout the test. It will be seen that the initial decline was about twice as great when the nerve was stimulated at 35 per second than when stimulated at about half that frequency. This harmonizes with Gerard's observations as to the correlation of the decrease in metabolic activity per impulse with the frequency of stimulation. It also appears from this table that the first response at low frequency after the end of high frequency stimulation, showed a slightly greater decline in those observations in which the low frequency was 35 per second. From this it seems possible that the relative refractory period was prolonged to approximately 30 σ after the previous response, and that part of the decreased size of the first response after the return to low frequency may be explained in this way. On the other hand, when the first response after cessation of the high frequency occurred between 20 and 25 σ after the previous response, the excursion was always between 93 and 99 per cent of the size of the next response, which occurred 62 σ later. Since recovery from the refractory period was so nearly complete in 20 σ, it must have been virtually complete after 62 σ. Therefore the lower level of response sustained for several successive impulses at 16 per second clearly indicates a more enduring depression.

We may reasonably identify this enduring depression with the "equilibration" fatigue discussed by Gerard. The question arises wherein it differs from the relative refractory period. Gerard (1927b) has offered reasons for postulating two distinct substances or sources of energy;—one used up in the single impulse and restored during the recovery process (relative refractory period), the other drawn on in the process of restitution and only gradually depleted after a prolonged series of impulses. To depletion of the latter is ascribed the reduced size of response indicative of fatigue. Our observations, showing the persistence of the lower level of response far beyond the limits of the relative refractory period, support Gerard's view and strongly suggest a different and more enduring kind of depletion from that observed during the recovery process following a single impulse.

With the highest stimulus frequencies employed in our experiments, 600 per second and over, responses occurred only to every other stimulus; that is, they occurred regularly at half the frequency of the stimuli, even at the beginning of stimulation. This simply means that the second stimulus fell in the refractory period following the first response, but the nerve was sufficiently recovered at the time of the third stimulus to respond again, etc. At these frequencies, on continued stimulation there was a tendency for the responses to become irregular. This effect was particularly striking in the first experiment, in which prolonged stimulation with various frequencies from 300 to 1000 per second were employed. Figure 9 shows six observations in this experiment with different frequencies of stimulation. At the lowest frequency (400 per second) the responses followed the stimuli at their full frequency at the beginning; in the other observations with frequencies of 750 and 1020, the nerve showed alternate responses at the beginning. In all cases, with continuance of stimulation the responses became highly irregular; after two or three seconds of stimulation the character of the record resembles that of a typical electromyogram in a sustained reflex or voluntary contraction of a muscle. In many of our records we find the same partial alternation described by Beritoff (1913), i.e., the full rhythm of stimulation is present but the responses appear alternately large and small. Beritoff interpreted this by assuming that the refractory phase set up by the second (subnormal) impulse was shorter than that set up by the first impulse, which was full-sized, and therefore the third stimulus, finding the nerve more fully recovered, evoked a larger response. This interpretation is rendered highly unlikely by our records in which regular stimulation with frequencies below 350 per second evoked regular responses of uniform size, although each occurred early enough in the relative refractory period to be decidedly subnormal. The inference is that as long as all the fibers are able to respond to every stimulus, the size of successive responses after the first will be the same, i.e., there will be no alternation of large and small responses in the individual fibers. The appearance of alternation, therefore, marks the failure of some fibers to respond to every stimulus. In a mixed nerve there is no reason to suppose that the refractory period of all the fibers will be the same, indeed the work

Fig. 9. Responses to high frequency stimulation, showing development of irregular rhythm; all from the same nerve, Dec. 5, 1928.

A and B 400 per sec.; B is continuation of A 1 sec. after beginning of stimulation.

C and D 750 per sec.; D, continuation of C 1.7 sec. after beginning.

E, 1020 per sec. A–E at speed of film shown by time record below E.

F and G 770 per sec.; G, continuation of F 1.75 sec. after beginning.

H, 1020 per sec. I, 770 per sec., record taken after 10 seconds of continuous stimulation.

F–I at speed of film shown by time marker at the bottom.

Magnification 410; string tension, 244 m. per amp.

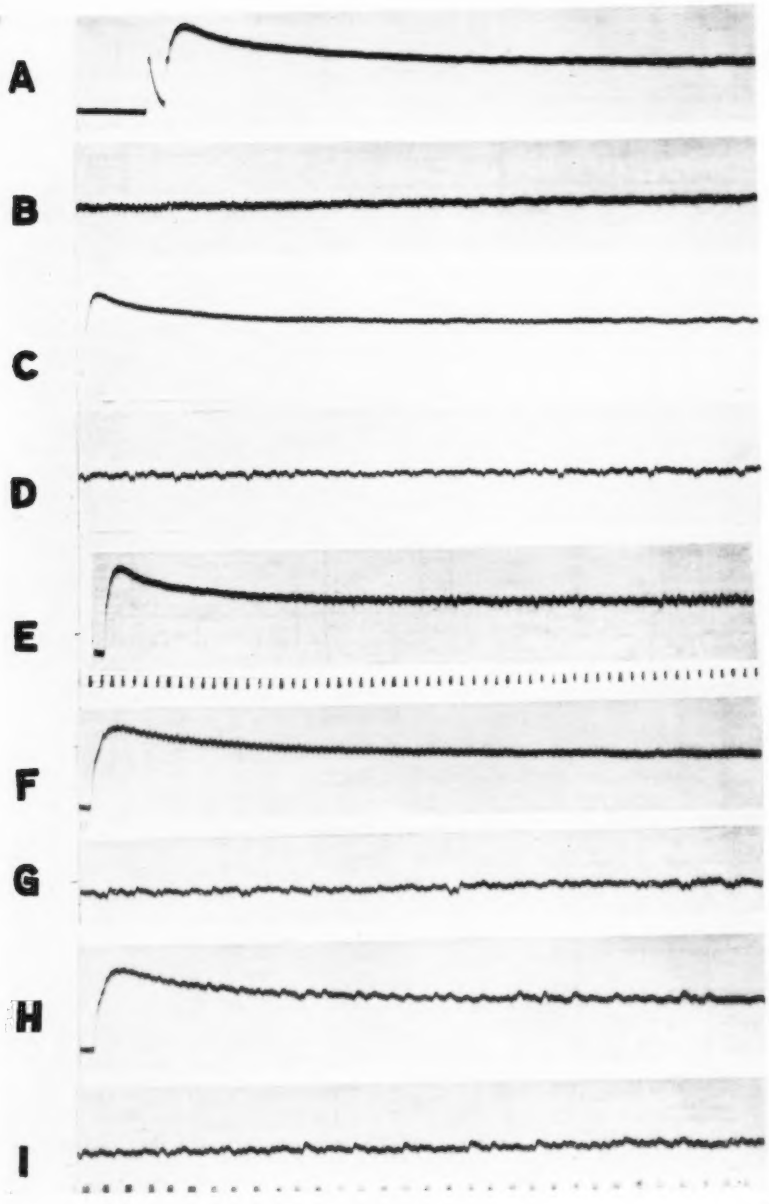

Fig. 9

of Erlanger, Bishop and Gasser (1926) shows definitely that there is a considerable range of variation between fibers. When the stimuli are just too frequent for some fibers to follow, but not too frequent for others, we find the regular rhythmic response to every stimulus on the part of the more rapid fibers and superimposed upon this the alternating response of the slower fibers. When the frequency is so high that even in the rested nerve very few of the fibers can respond to every stimulus, and none can follow the stimulus frequency when the refractory period has become prolonged in consequence of continued stimulation, irregularity begins to appear. It becomes a statistical matter of chance what percentage of fibers are sufficiently recovered to respond to any given stimulus. The result is that the response of the nerve as a whole presents the highly irregular picture shown in figure 9. The regularity of the stimuli is well controlled by the records made at many different frequencies in nearly all of our experiments (see fig. 2). We have here an interesting example of the irregularity of response of living material to stimuli applied in perfect rhythm.

SUMMARY

1. A new apparatus, described by Campbell, for electrical stimulation, operating by condenser discharges controlled by an electron tube, provides rhythmic stimuli of great regularity at any frequency up to more than 1000 per second. It also makes possible an instantaneous change of frequency over a very wide range.

2. This apparatus has facilitated a study of the effects of high frequency stimulation on the action current of nerve.

3. During the continuance of high frequency stimulation the successive responses become gradually smaller, the decline being most rapid in the first quarter of a second, but continuing to be appreciable for many seconds. The possible dependence of this effect on local "stimulation fatigue" was controlled by shifting the stimuli to a different pair of electrodes. The effect was thus found to depend on the conducted impulses.

4. The decrease in size of response during high frequency stimulation might depend merely on the prolongation of the relative refractory period; therefore stimuli were applied to the nerve at frequencies so low that each should find the nerve fully recovered from the refractory period, and the responses evoked at these low frequencies before and after high frequency stimulation were compared. Again the possibility of confusion due to stimulation fatigue was controlled by applying the test stimuli at electrodes distal to those used to produce fatigue. It was found that after 20 to 25 seconds of stimulation at frequencies between 200 and 400 per second, the size of response fell to about 75 per cent of its initial value.

5. When a nerve is stimulated with a higher frequency than the responses

can follow, the rhythm of response is often half of the stimulus rhythm. When the stimulus frequency is just below the upper limit of response frequency at the outset, continued stimulation often results in responses appearing alternately large and small. Prolonged stimulation at these higher frequencies results in irregular responses. Both of these effects are presumably due to differences between the refractory periods of the individual fibers.

Miss Rice performed a large and important part of the experiments here recorded. Her untimely death on April 1, 1929, prevented her participation in the final preparation of the work for publication.

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BIBLIOGRAPHY

Adrian, E. D. 1912. Journ. Physiol., xlv, 389.
Adrian, E. D. and A. Forbes. 1922. Journ. Physiol., lvi, 301.
Beritoff, J. S. 1913. Zeitschr. f. Biol., lxii, 125.
Bishop, G. H. 1928. This Journal, lxxxiv, 417.
Bishop, G. H. and J. Erlanger. 1926. This Journal, lxxviii, 630.
Bramwell, J. C. and K. Lucas. 1911. Journ. Physiol., xlii, 495.
Campbell, C. J. 1929. This Journal, lxxxix, 443.
Davis, H. and Brunswick. 1926. This Journal, lxxv, 497.
Davis, H., A. Forbes, D. Brunswick and A. McH. Hopkins. 1926. This Journal, lxxvi, 448.
Erlanger, J., G. H. Bishop and H. S. Gasser. 1926. This Journal, lxxviii, 537.
Field, H., Jr. and E. Th. Brücke. 1926. Pflüger's Arch., ccxiv, 103.
Forbes, A., L. H. Ray and F. R. Griffith, Jr. 1923. This Journal, lxvi, 553.
Forbes, A. and A. Gregg. 1915. This Journal, xxxvii, 118.
Forbes, A. and D. W. Mann. 1924. Journ. Optical Soc. of America, viii, 807.
Gasser, H. S. and J. Erlanger. 1925. This Journal, lxxiii, 613.
Gerard, R. W. 1927a. Journ. Physiol., lxiii, 280.
1927b. Science, lxvi, 495.
Gerard, R. W. and A. Forbes. 1928. This Journal, lxxxvi, 178.
Gotch, F. 1910. Journ. Physiol., xl, 250.
Kato, G. 1924. The theory of decrementless conduction in narcotised region of nerve. Tokyo.
Tigerstedt, C. 1912. Zeitschr. f. Biol., lviii, 451.

A PLETHYSMOGRAPHIC STUDY OF CHANGES IN THE VOLUME OF THE LIVER IN THE INTACT ANIMAL

HAMLINE MATTSON

From the Division of Experimental Surgery and Pathology, The Mayo Foundation, Rochester, Minnesota

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Although changes in volume of various organs have been studied for many years with profitable results, such studies on internal viscera have been conducted, until recently, as experiments on acute changes. It is obvious that such studies, complicated by the anesthetic and operative procedures, would not be most suitable for detecting changes in the volume of an organ over extended periods of time and changes dependent on the emotional state of the animal. To circumvent such objections, Hargis and Mann devised, and used with success, a collodion plethysmograph for the spleen. The plethysmograph is applied aseptically and the study is conducted later when the animal has recovered. This paper is a record of the technic evolved for plethysmography of a lobe of liver and a study of the changes in the volume of the liver in the intact animal.

The work may be divided into: 1, the development of a method for plethysmography of a lobe of liver; 2, a study of the changes in the volume of the liver under normal and varying emotional conditions; 3, the effect of administration of various substances, such as amyl nitrite, epinephrin, solutions of peptone and sugar,[1] chloroform and synthalin, and 4, the effect of such acts as rebreathing and vomiting on the volume of the liver.

REVIEW OF LITERATURE. François-Franck and Hallion (11) first recorded changes in the volume of the liver. They investigated the effect of circulatory and nervous influences on the volume of the liver and concluded that hepatic vasomotor nerves branch off from the sixth dorsal to the second lumbar levels (12, 13, 14). Thompson found that the liver was more sensitive to injection of peptone and proteoses, responding with an increase in volume, than were the intestines, spleen, kidney and limbs. Thacher induced venous stasis by introducing a balloon near the heart and found that the liver, small intestines, spleen and extremities decreased in

[1] The work with amyl nitrite, epinephrin, peptone and sugar is a repetition on the intact animal of previous work in acute experiments and affords a control of the method and a comparison between the results obtained under the conditions of my experiments as compared with those of other workers.

volume. Neubauer (31) punctured the floor of the fourth ventricle in a study of the mechanics of glycosuria in rabbits and found that the volume of the liver increased. Increases in the volume of the liver were recorded in rabbits following intravenous injection of epinephrin, pituitrin and strophanthin; the volume was increased in asphyxia by clamping the trachea, stimulation of the central end of the vagus and of the central end of the splanchnic nerves (32). Decrease in volume followed the administration of extract of anterior hypophysis, veronal, ergotoxin, by bleeding, by the application of heat and cold to the skin, and by the stimulation of the peripheral end of the vagus. Edmunds (4) recorded an initial decrease and then an increase in the volume of the liver in anaphylaxis in dogs and cats. Epinephrin (5) and amyl nitrite (6) decreased the volume of the liver. Edmunds and Stone recorded an initial decrease, then a great increase in the volume of the liver in rabbits after the injection of epinephrin. Mautner and Pick (28) found that the volume of the liver increased in shock from peptone or histamine, and in anaphylactic shock in carnivora. The outflow from the hepatic vein decreased as the liver increased in volume and increased again as the liver decreased (29). Epinephrin partially shut off the inflow and outflow through the hepatic veins as it decreased the volume of the liver. Sodium nitrite, and in a less degree papaverine, counteracted such effects. Pulse changes in the volume of the liver synchronous with the pulse were noted (30). Mautner (26) presented evidence to show that the vessels of the liver play an important part in regulating the water balance in the tissues. The injection of glucose, levulose and maltose (27) was followed by increases in the volume of the liver. The administration of glucose did not have any effect on a dehydrated animal. Lactose, mannose and saccharose did not have any effect. Jackson recorded decreases in the volume of the liver following the administration of epinephrin and tetramethylammonium chloride. Bainbridge and Trevan placed a small balloon between two lobes of the liver and connected it to a recorder, thus recording increases in the volume of the liver in dogs and cats after epinephrin had been injected. Hunt found that acetyl cholin and histamine decreased the volume of the liver. Elias and Sammartino also recorded increases in the volume of the liver in rabbits after the injection of epinephrin. Lampe and Méhes (22) obtained diminished outflow and a decrease of volume in isolated livers of dogs, cats, and to a less extent in rabbits, after the injection of epinephrin. Decreases in the volume of the liver and outflow from the hepatic vein followed injection of nicotine, barium chloride, saponin, and physostigmine (23). Papaverine had the opposite effect. Baer and Rössler reported an increase in the volume of the liver and decreased outflow following injection of histamine. McLaughlin obtained decreases in the volume of the liver and in the outflow from the hepatic vein following the injection of epinephrin,

pituitary extract and barium chloride. Sodium nitrite had the opposite effect.

METHOD OF STUDY. Even in experiments concerned with acute changes in volume, in which a plethysmograph of sheet brass or similar material is applied, a number of conditions are encountered which make plethysmography of this organ difficult. The most important of such conditions are the relative inaccessibility of the organ and the movements of the diaphragm. The method described in this paper, although designed for experiments on intact animals, is suitable for experiments on acute changes. The type of exposure adopted has been largely responsible for the success attained.

Paraffin models of various sizes are molded to conform to the shape of the left lateral lobe of the liver in the dog. A glass tube is inserted on the diaphragmatic surface as indicated (fig. 1). A plethysmograph is then made of successive layers of collodion and gauze applied to the model of the lobe of liver in the same manner as described by Hargis for making a plethysmograph for the spleen. Several plethysmographs of various sizes should be prepared because of the variations in size of the left lateral lobe of the liver. A rubber tube connects the inside of the plethysmograph with the recording apparatus (fig. 1).

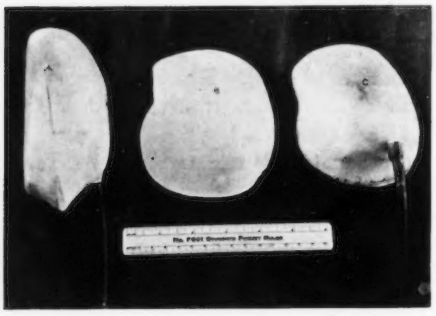


Fig. 1. Plethysmograph, showing place where glass and rubber tube are attached.

The plethysmographs are sterilized by boiling. Utmost care must be used in introducing a foreign body into the abdomen. Mann has observed that as little as 1 gram of hepatic tissue for each kilogram of body weight, if left in the abdominal cavity without circulation, may cause death within twenty-four hours. Such an observation emphasizes the importance of reducing injury to the liver to a minimum in the intact animal. The animals recover readily from tears in the liver, but manual pressure on the lobe incident to poor exposure may cause death in a few hours.

All operative procedures were carried out under complete anesthesia with tracheal insufflation and rigid sterile technic. The left lateral lobe of the liver is exposed best by a thoraco-abdominal approach. The left pleural cavity is opened through the eighth intercostal space (fig. 2 *b*). All structures except the diaphragm are packed off (fig. 2). The diaphragm then is incised parallel to its muscle fibers and the incision carried through

the costal cartilages and upper abdominal wall. This gives good exposure of the desired portion of the liver. All structures except the left lateral lobe and its pedicle are packed off. Peritoneal reflections may be cut to free the pedicle. A collar of great omentum is applied around the pedicle and an apron of omentum spread over the lobe (fig. 2 *a*). A plethysmograph of suitable size is selected and an aperture for the pedicle is cut to fit. Care must be taken to prevent compression of the blood vessels to the lobe.

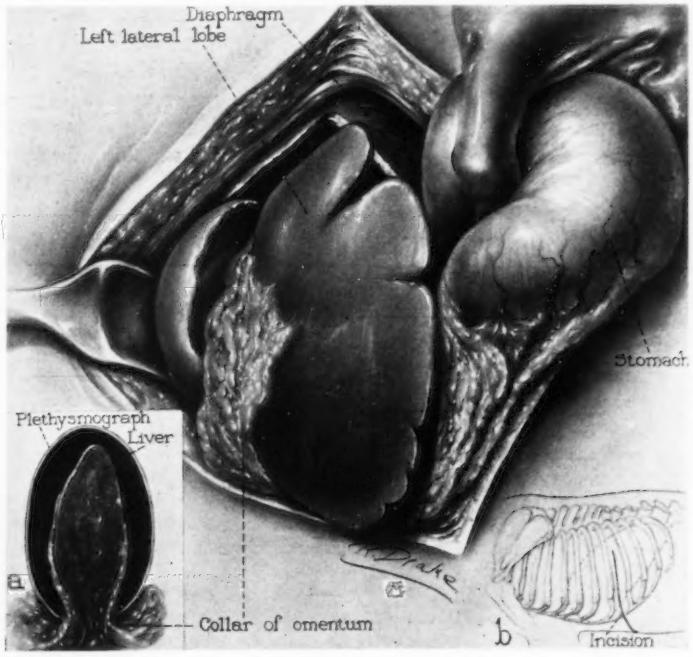


Fig. 2. Exposure and isolation of left lateral lobe of liver in the dog: *a*, longitudinal section showing relative portions of lobe of liver, collar of omentum, and plethysmograph; *b*, line of primary incision.

The lobe and the omental apron are slipped carefully into the plethysmograph. The opening in the plethysmograph is closed with double locked linen sutures. Available omentum is sewed on the stomach where that organ comes in contact with the plethysmograph. The suture line of the plethysmograph is sealed with a layer of gauze and collodion. The rubber tube is slipped through an abdominal stab wound. The incision in the diaphragm is sutured and the thoracic cavity is closed in the usual manner.

When observations are to be made, the animal is laid on its right side on

a padded holder. A collar is applied about its neck and the feet are restrained gently. The tube is connected with a water manometer, or a Brodie bellows recorder, and is tested for leaks. Records also may be obtained according to the method developed by Potter. The trained dog will lie comfortably and quietly for a long time, sometimes sleeping throughout the observations.

Amyl nitrite and chloroform were inhaled directly from a sponge. Other substances were injected into the external jugular and saphenous veins with as little manipulation as possible. The carbon dioxide content of the blood was increased with a mask of a type employed as a routine in metabolism work in the laboratory. This mask is described briefly by Boothby and Sandiford and in detail by Kitchen. The mask was connected to a closed system of 20 liters capacity which contained a rubber balloon to care for the respiratory excursions. The neck of the dog was shaved, the mask fitted in place, and a wide dental dam was wrapped around the neck of the dog and the posterior portion of the mask until the joint was airtight. Respiratory movements were recorded in the usual manner.

Fig. 3. Effect of inhalation of amyl nitrite on the volume of the liver. Amyl nitrite was inhaled twice at arrow. (In this and succeeding figures the time marker indicates seconds.)

Fig. 4. Effect of varying concentrations of epinephrin on the volume of the liver. At the first arrow the respiratory tambour was connected and 1 cc. of 1:25,000 epinephrin was injected rapidly into the left saphenous vein. The second arrow indicates reinsertion of the needle and syringe containing 1 cc. of 1:50,000 epinephrin. Rapid injection was made at the third arrow. The needle was reinserted at the fourth arrow, and 1 cc. of 1:100,000 epinephrin was injected rapidly at the fifth arrow.

Fig. 5. Effect of epinephrin in larger doses without vomiting. The needle was inserted at the first arrow; 0.5 cc. of 1:1000 epinephrin was injected rapidly at the second arrow. The time from the second arrow to the return to the former level was about eleven minutes.

Fig. 6. Effect of injection of Witte's peptone on the volume of the liver. The needle was inserted at *1*. Injection of 12 cc. of 6 per cent peptone in distilled water in left external jugular vein began at *2* and ended at *3*. Movements of the animal took place at *4*. The pointer went off the drum at *5* and *6*, but an attempt was made to record amplitude of excursions. The pointer went off the drum at *7* and *8*, but an attempt was made to record its excursions.

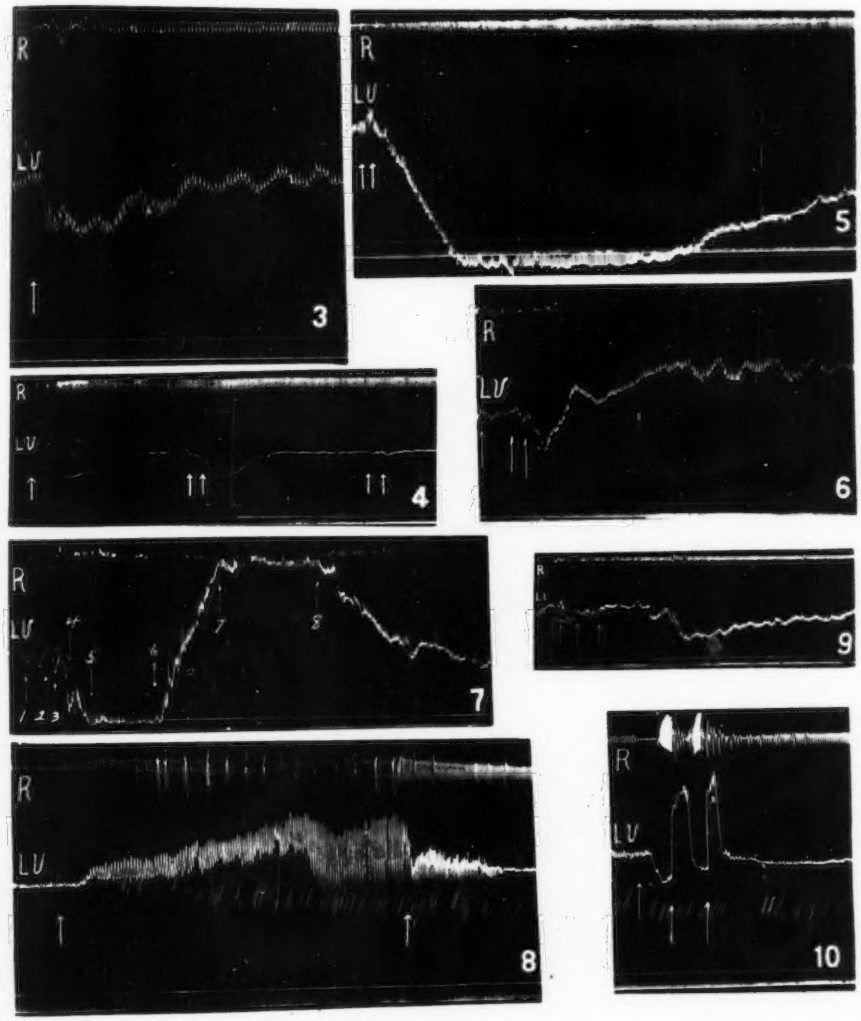
Fig. 7. Effect of injection of glucose on the volume of the liver. The needle was inserted in the left saphenous vein at the first arrow, and 50 cc. of 30 per cent glucose was injected between the second and third arrows.

Fig. 8. Effect of rebreathing. The system into which the animal breathed was closed at the first arrow and opened at the second arrow.

Fig. 9. Effect of chloroform inhalation on the volume of the liver. Chloroform-soaked gauze was placed near the animal's nose at the first arrow. The animal moved at the second and third arrows. The gauze was removed at the fourth arrow.

Fig. 10. Effect of vomiting. At the first arrow 0.5 cc. of 1:1000 epinephrin was injected in the left saphenous vein. The second and third arrows mark beginning of acts of vomiting.

RESULTS. *Normal changes in the volume of the liver and the effect of environmental stimuli.* It was noticed early that when complete quiet and rest were obtained there was practically no change in the volume of the liver except small variations synchronous with respiratory movements



Figs. 3 to 10

and the pulse. Increases were recorded during inspiration and decreases during expiration. However, if a door was opened suddenly, or if some one walked about the room or talked after a quiet period, more pronounced variations in the volume of the liver were recorded. A slight increase and then a more pronounced decrease followed directly on the excitation. Such variations continued beyond the initial one and were present following the administration of various substances (figs. 3 to 7).

Inhalation of amyl nitrite. A 5 minim vial of amyl nitrite was broken in gauze and placed near the nose of the animal for two inspirations. Rapid decrease in the volume of the liver was recorded (fig. 3). The volume of the liver returned to its former level in forty to eighty-five seconds.

Effect of ephinephrin. This drug caused rapid and pronounced decrease in the volume of the liver in each case when injected intravenously. It had very little effect in a strength of 1 cc. of 1:100,000 solution (fig. 4). One cubic centimeter of 1:50,000 epinephrin caused a decrease in the volume of the liver as great as that produced by the 1:25,000 strength, but the period of return to the original volume was half as long with 1:50,000 strength. Still larger doses produced proportionately greater decreases in the volume of the liver and proportionate prolongation of recovery, except when vomiting was induced. In a dog that did not vomit after 0.5 cc. of 1:1000 epinephrin had been given, a marked decrease in volume took place over a period of about a minute. The decrease persisted for five minutes. Seven minutes were required for the volume to return to the original level (fig. 5). Small amounts of epinephrin, such as are used in injections of cocaine in dental surgery, caused slight, delayed and prolonged decrease in the volume of the liver. Less marked decreases were recorded in dogs when the portal blood was shunted to the inferior vena cava through a true Eck fistula.

Peptone shock. Witte's peptone, in a dosage of 0.01 gram for each kilogram of body weight, produced a slight decrease in the volume of the liver when injected in the left saphenous vein. In another animal 0.05 gram for each kilogram produced a marked reaction, with prostration irregular, rapid and sometimes deep respirations, pallor of the mucous membranes and spasms of the muscles of the neck. The eye reflex was present, but the animal would not respond to pressure stimuli. The volume of the liver decreased greatly at first and then increased (fig. 6). The reaction began as the liver decreased in volume and subsided as it returned to the usual level.

Effect of the intravenous injection of sugars. Glucose (fig. 7) and levulose caused definite increases in the volume of the liver when injected intravenously; these increases were greater than the increases caused by injection of equal amounts of isotonic solution of sodium chloride.

Effect of rebreathing. The amplitude of the changes synchronous with

respiratory movements increased with rebreathing and a gradual increase in the volume of the liver was recorded (fig. 8).

Effect of chloroform. Gauze was soaked with chloroform and placed above and near the animal's nose. A rapid decrease in the volume of the liver was noted and the return to the original volume was prolonged. Sometimes the volume of the liver decreased still further after an interval of a few seconds in some cases and up to a minute or two in others (fig. 9). A decrease in the volume of the liver was recorded following inhalation of chloroform in a dog in which the portal blood was shunted into the inferior vena cava in a true Eck fistula.

Synthalin. A decrease in the volume of the liver followed by a slow return to the original volume was recorded following intravenous injection of synthalin in a dosage of 10 mgm. for each kilogram of body weight. The effect simulated that of chloroform.

Effect of vomiting. A period of vomiting of short duration was induced by injection of 0.5 cc. of 1:1000 epinephrin. A gradual increase in the volume of the liver was recorded in the periods directly preceding vomiting, but in the act of vomiting itself a marked and rapid increase in the volume of the liver was recorded (fig. 10).

COMMENT. Although the results obtained on the changes in volume of the liver in the intact animal are quite definite, it is apparent that several possible sources of error must be ruled out before the results may be considered of physiologic significance. The effects of the movements of the animal and the excursions of the diaphragm are significant. Only animals trained for the purpose were used and movements were reduced to a minimum. When movements did occur they were shown on the record. Recording respiratory movements coincident with changes in the volume of the liver shows that pronounced changes in volume take place when respiration is unaltered, and vice versa. It is possible that respiratory movements cause changes in the volume of the liver not only by compression of the organ but by the effect of the respiratory pump on the great veins. Direct influence of the wall of the chest on the plethysmograph and alteration in the position of the pedicle of the lobe of the liver by excursions of the diaphragm must be considered as factors also.

A study of plethysmographic records showed that the volume of the liver was variable, depending not only on pulse and respiration, but on the physiologic state of the animal. The rhythmic changes following such stimuli as sudden sound and other procedures are approximate in amplitude to the spontaneous changes recorded in the spleen of the intact animal by Hargis and Mann. They were eliminated when the animal was placated and conversely they were numerous when the animal was excited. It may be true that this response is general to all the viscera innervated by the splanchnic nerves, since Hargis and Mann have shown that section of the

nerves to the spleen abolishes the so-called splenic reflex. James stated: "The bodily changes follow directly the perception of the exciting fact and our feeling of the same changes as they occur is the emotion." The record of Hargis and Mann concerning the spleen and my records concerning the liver in the intact animal may be considered as suggestive evidence supporting this theory of the emotions.

The changes recorded following the administration of drugs in my experiments are similar to the results obtained in acute experiments. This observation confirms the reliability of the older work on the volume of the liver.

The probable part of the liver as a reservoir for blood has received little attention since Stolnikow's experiments in 1882. Valves are absent between the sinus and auricles of the heart in birds and mammals. The first valves to be encountered on the venous side are in the femoral, subclavian and jugular veins. The right side of the heart, therefore, opens directly into a venous cistern composed of the vena cava, and the hepatic, innominate, iliac, and renal veins. The hepatic veins are the largest tributaries of the vena cava. Their branches stand open when cut. In vomiting, the column of blood is dammed back into the venous cistern and the patent hepatic veins. Similar mechanical factors operate in respiration and coughing. The observation that the volume of the liver increases with inspiration and decreases with expiration confirms an earlier observation in acute experiments by Neubauer (31). Other workers on anesthetized animals recorded changes in the volume of the liver synchronous with respiration, including Jackson who used a brass plethysmograph. Schmid observed slowing of the blood stream and an increase in blood pressure in the portal vein during inspiration. At the beginning of expiration or soon after, the flow of blood in the portal vein increased and the portal pressure decreased. When intrabronchial pressure was raised, ebb in the portal vein and increase in portal pressure followed. Respiratory variations in the volume of the liver have been found to be absent in some of the records taken on anesthetized animals. I found them always present in some degree. The lobe of the liver and the plethysmograph were excised in toto at necropsy in a successful preparation, and the plethysmograph was connected to a manometer. Tests showed it to be airtight. Manual manipulation of the pedicle and pressure on the plethysmograph did not cause material change in the level of the manometer. The evidence minimized the importance of movements of the wall of the chest and the diaphragm as factors in this study.

The control which the liver may exert over the blood flow through it has received scant attention. The potential significance of such control is tremendous. The superior vena cava or the inferior vena cava below the hepatic veins may be ligated without affecting systemic blood pressure

materially but when this hepatic reservoir with the portal system is cut off from the heart there follows a rapid and marked decrease of pressure in the carotid artery (9). Flack and Hill (10) have noted that blocking the portal veins so congests the abdominal vessels that they can hold nearly all the blood in the body; the arterial pressure decreases rapidly, and the animal dies. Accumulating evidence is leaving less support for the old view that the liver is a passive sluice for portal blood. The nerve trunks entering the liver are significantly large. Definite vasomotor action in the liver has been proved. Huber found that the muscle in the portal radicles disappears rapidly but there is considerably more in the sublobular veins. Mautner and Pick (28, 29) Lampe and Méhes and Baer (22, 23) and Rössler, McLaughlin and others have shown that the vessels of the liver exercise definite control over the blood flow in the hepatic vein in perfusion experiments. Perhaps the mechanical rôle which this, the largest organ in the body, plays is commensurate with its size.

SUMMARY

Literature of changes in the volume of the liver in anesthetized animals is reviewed. A method is noted whereby changes in the volume of the liver may be recorded in the intact dog. Changes in the volume of the liver associated with emotions and changes synchronous with respiratory movements and the pulse were observed.

Decreases in the volume of the liver are recorded following inhalation of amyl nitrite and the injection of epinephrin. Increases are recorded after the injection of glucose and levulose. An initial decrease and then an increase in the volume of the liver is recorded following injection of peptone. The results with amyl nitrite, epinephrin, glucose, levulose, and peptone simulate effects in previously recorded work on anesthetized animals. An increase in the volume of the liver is recorded in rebreathing. Intravenous injection of synthalin causes a decrease in the volume of the liver as does inhalation of chloroform. Rapid increases in the volume of the liver are recorded during vomiting.

BIBLIOGRAPHY

(1) Baer, R. and R. Rössler. Wien. klin. Wochenschr., 1926, xxxix, 1033.
(2) Bainbridge, F. A. and J. W. Trevan. Journ. Physiol., 1917, li, 460.
(3) Boothby, W. M. and I. Sandiford. This Journal, 1923, lxvi, 93.
(4) Edmunds, C. W. Zeitschr. f. Immunitätsforsch. u. exper. Therap., 1914, xxii 181.
(5) Edmunds, C. W. Journ. Pharm. Exper. Therap., 1914–1915, vi, 569.
(6) Edmunds, C. W. Journ. Pharm. Exper. Therap., 1921–1922, xviii, 155.
(7) Edmunds, C. W. and R. P. Stone. Arch. internat. de pharmacol., 1923–1924, xxviii, 391.
(8) Elias, H. and U. Sammartino. Biochem. Zeitschr., 1921, cxvii, 10.

(9) FLACK, M. AND L. HILL. A textbook of physiology. London, Longmans, Green and Co., 1919, p. 162.
(10) FLACK, M. AND L. HILL. A textbook of physiology. London, Longmans, Green and Co., 1919, p. 247.
(11) FRANÇOIS-FRANCK, CH.-A. AND L. HALLION. Arch. de physiol. norm. et path., 1896, viii, s. 5, 908.
(12) FRANÇOIS-FRANCK, CH.-A. AND L. HALLION. Arch. de physiol. norm. et path., 1896, viii, s. 5, 923.
(13) FRANÇOIS-FRANCK, CH.-A. AND L. HALLION. Arch. de physiol. norm. et path., 1897, ix, s. 5, 434.
(14) FRANÇOIS-FRANCK, CH.-A. AND L. HALLION. Arch. de physiol. norm. et path., 1897, ix, s. 5, 448.
(15) HARGIS, E. H. Journ. Lab. Clin. Med., 1926, xi, 664.
(16) HARGIS, E. H. AND F. C. MANN. This Journal, 1925, lxxv, 180.
(17) HUBER: Quoted by EDMUNDS (5).
(18) HUNT, R. This Journal, 1918, xlv, 197.
(19) JACKSON, D. E. Experimental pharmacology. St. Louis, C. V. Mosby Co., 1917, p. 422.
(20) JAMES, W. The principles of psychology. New York, Henry Holt and Co., 1890, ii, 449.
(21) KITCHEN, H. D. This Journal, 1924, lxvii, 487.
(22) LAMPE, W. AND J. MÉHES. Arch. f. exper. Path. u. Pharm., 1927, cxix, 66.
(23) LAMPE, W. AND J. MÉHES. Arch. f. exper. Path. u. Pharm., 1927, cxix, 73.
(24) MCLAUGHLIN, A. R. Journ. Pharm. Exper. Therap., 1928, xxxiv, 147.
(25) MANN, F. C. Current Res. Anesth. and Anal., 1925, iv, 107.
(26) MAUTNER, H. Wien. Arch. f. inn. Med., 1923, vii, 251.
(27) MAUTNER, H. Arch. Exper. Path. u. Pharm., 1927, cxxvi, 255.
(28) MAUTNER, H. AND E. P. PICK. München. med. Wochenschr., 1915, lxii, 1141.
(29) MAUTNER, H. AND E. P. PICK. Biochem. Zeitschr., 1922, cxxvii, 72.
(30) MAUTNER, H. AND E. P. PICK. Arch. f. exper. Path. u. Pharm., 1923, xcvii, 306.
(31) NEUBAUER, E. Biochem. Zeitschr., 1912, xliii, 335.
(32) NEUBAUER, E. Biochem. Zeitschr., 1914, lii, 118.
(33) POTTER, J. C. Journ. Lab. Clin. Med., 1924, ix, 648.
(34) SCHMID, J. Pflüger's Arch., 1909, cxxvi, 165.
(35) STOLNIKOW. Pflüger's Arch., 1882, xxviii, 255.
(36) THACHER, H. C. Deutsch. Arch. f. klin. Med., 1909, xcvii, 104.
(37) THOMPSON, W. H. Journ. Physiol., 1899, xxv, 1.

CHANGES IN THE VOLUME OF THE KIDNEY IN THE INTACT ANIMAL: A PLETHYSMOGRAPHIC STUDY WITH ESPECIAL REFERENCE TO DIURETICS

W. L. REID[1]

From the Division of Experimental Surgery and Pathology, The Mayo Foundation, Rochester, Minnesota

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Changes in the activity of an organ are usually associated with alterations in blood flow as well as with the specific response of the parenchyma to a given stimulus. Changes in volume of an organ result from alterations in blood flow and from the elimination or temporary retention of products of activity of the organ. Although these two curves do not always coincide or parallel each other, they are often similar, as has been shown by other investigators. Many studies on the variation in volume of the kidney have been made since Roy invented the oncometer (1881). However, previous studies of renal volume have been made only on anesthetized or decerebrated animals, or on perfused kidneys, and the inhibitory effects of general anesthesia and surgical procedures on the function of the kidney, although temporary, are quite marked. General anesthesia decreases the functional capacity of the kidney for a considerable period of time and the mere handling of a kidney during an operation is often sufficient to produce temporary anuria. As pointed out by Schäfer and Herring the anesthetic and the operative procedure in applying a renal oncometer frequently combine to produce suppression of urine throughout the experiment.

In view of these facts, I began a study of changes in renal volume in the intact animal, with especial reference to the action of the more common diuretics. The value of such a study lies in the fact that it not only gives a clue to the activity of the kidney, but lends additional data on the pharmacologic action of certain drugs on that organ.

In working out the problem it was necessary to use a plethysmograph which could be left to be healed in situ after its application. Such an instrument was found in the collodion plethysmograph of Hargis, and used by Hargis and Mann in their studies on changes in splenic volume in the intact animal. This plethysmograph is light, durable, rigid, easily applied,

[1] Fellow in Surgery.

and non-irritating. The method which is to be described is essentially an adaptation of the use of this plethysmograph.

In making plethysmographic study of an organ in situ it is obvious that the coöperation of the animal during observation is necessary if accurate results are to be obtained. Dogs, therefore, were selected for experimentation because they are easily trained and because the kidney of this species is anatomically well adapted for plethysmographic study.

Models of normal dog kidneys of various sizes were fashioned from warm paraffin, with especial care to insure perfectly smooth and uniform surfaces (fig. 1 *a*). A short piece of glass tubing, 8 mm. in diameter, was inserted into each model on the dorsal edge, almost directly opposite the hilum. The paraffin then was hardened by cooling, and the model was covered with four to six single layers of gauze. Each layer of gauze re-

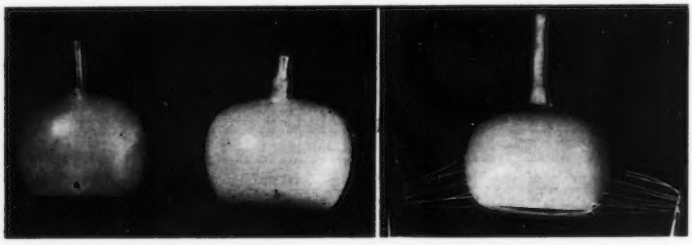

Fig. 1 Fig. 2

Fig. 1. *a*, Lateral view of a paraffin model of a normal kidney; *b*, lateral view of a plethysmograph prepared on the model; the paraffin has not yet been removed.

Fig. 2. The plethysmograph ready for sterilization. Paraffin has been removed, the rubber tube has been applied, and the interrupted sutures have been placed in situ.

ceived three or four coats of non-flexible collodion which were rubbed on vigorously before the succeeding layer was applied. The glass tube was covered in a similar manner and was carefully reënforced at its juncture with the body of the plethysmograph to prevent binding or breaking at that point (fig. 1 *b*). After the final coat of collodion, the model was allowed to dry in a cool place for eighteen hours. The paraffin then was removed by incising the ventral edge longitudinally and boiling the instrument in water for ten or fifteen minutes. When the collodion was applied correctly "blisters" did not result. The glass tube next was removed and a piece of firm rubber tubing, 10 cm. in length, was fastened on the plethysmograph to serve as a connection between the latter and the recording apparatus. In order that the instrument might be applied without injury to the kidney, the ventral opening in it was enlarged by extending the incision the necessary distance at each end. Finally, a suitable aperture for

the renal vessels and ureter was made and three or four interrupted linen sutures for closing the plethysmograph were placed in each end and secured with artery forceps (fig. 2). The instrument then was sterilized before operation by boiling it in water for twenty minutes.

Because of ease of approach, the left kidney was selected for experimentation. All operative procedures were performed under ether anesthesia, using a rigidly sterile technic. Through a left rectus incision the kidney was exposed and delivered into the wound. It was then covered with the most avascular and fat-free portion of the omentum, taking care to avoid any tension on the latter. A plethysmograph approximately a fourth larger than the kidney was applied and the instrument was closed by tying the interrupted linen sutures. To prevent leakage, the edges were sealed with gauze strips and collodion, which was allowed to dry for several minutes. A portion of the remaining omentum then was reflected around the entire plethysmograph and gently sutured in place, after which the kidney, with its plethysmograph, was returned to its normal position. A suitable stab wound was made lateral to the incision and adjacent to the kidney, through which the rubber tube was drawn. The abdomen then was closed in the usual manner.

Observations may be made as soon as the general condition of the animal permits, often as early as the second or third post-operative day. During the convalescent period, the dogs easily were trained to remain quietly on the observation table for several hours at a time. In each experiment, the animal was placed on his right side, on a padded concave holder, with a collar fastened about the neck, and the feet loosely restrained. The plethysmograph then was connected with a sensitive piston recorder, selected for measuring the changes in volume. The size of the piston recorder selected for each experiment depended on the size of the kidney as well as on the amplitude of pulsations (pulse pressure) of the renal artery. Consistently clear and accurate kymographic records were obtained.

The application of this method enables one to study changes of volume of the kidney in the virtually intact animal without the complications accompanying the acute experiments. During this investigation the kidney was studied by this method both in the intact state and after denervation. In addition, the influence of diuretics and other substances and procedures which affect the flow of urine were studied in their relation to changes of renal volume in the virtually intact animal. With the skilful use of omentum, this method, of itself, does not require an interval of time before experimentation. The plethysmograph, if applied correctly to the kidney, may be used immediately in an acute experiment, and when necessary without closing the abdomen.

METHOD OF EXPERIMENTATION. In many experiments, blood pressure was recorded simultaneously with renal volume. This was accomplished

under local anesthesia, with the use of sterile operative technic. A cannula was inserted into the carotid in the usual manner, and a 4 per cent solution of heparin in Ringer-Locke's solution was used in the tubing to prevent clotting. At the end of the experiment, the skin was anesthetized again and then closed loosely.

Changes of renal volume were studied in relation to the following diuretics: caffeine, theobromine sodio-salicylate (diuretin), theophyllin-ethylene diamine (euphyllin), pituitrin, digitalis, merbaphen, and also isotonic and hypertonic solution of sodium chloride and of glucose. Observations also were made on the effect of epinephrin, the nitrites, and distilled water. In addition, the reflex response to different stimuli, and the influence of spinal anesthesia on the renal volume were noted. All drugs and solutions were administered intravenously, and each experiment was repeated four or five times in order to determine that coördinate results could be obtained. Spinal anesthesia was obtained by the injection of 5 cc. of 3 per cent solution of procaine at the level of the sixth thoracic vertebra.

RESULTS. *Spontaneous changes.* As observed by Hargis and Mann, definite but small variations of renal volume occurred in the absence of stimuli, and without any concomitant changes of respiration or blood pressure. These changes obviously were of vasomotor origin. However, under the conditions of observation they were slight as well as inconstant, and therefore were disregarded during the remaining experiments.

Caffeine. The drug was administered in two forms, caffeine citrate and

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Fig. 3. Effect of caffeine. In this as in all subsequent tracings, *R* represents respiration; *B.P.*, blood pressure; *K.V.*, volume of kidney; *B*, base line; *T*, time in intervals of four seconds. There was an increase in renal volume following the injection of the drug.

Fig. 4. Effect of theophyllin-ethylene diamine. There was an initial decrease in renal volume followed by a marked and sustained increase.

Fig. 5. Effect of a small dose of pituitrin. There was marked immediate increase in renal volume. Comparison of this figure should be made with figure 6.

Fig. 6. Effect of a large dose of pituitrin. There was marked initial decrease in renal volume followed by sustained increase.

Fig. 7. Effect of merbaphen in renal volume. There was slight initial decrease in renal volume, followed by marked and sustained increase.

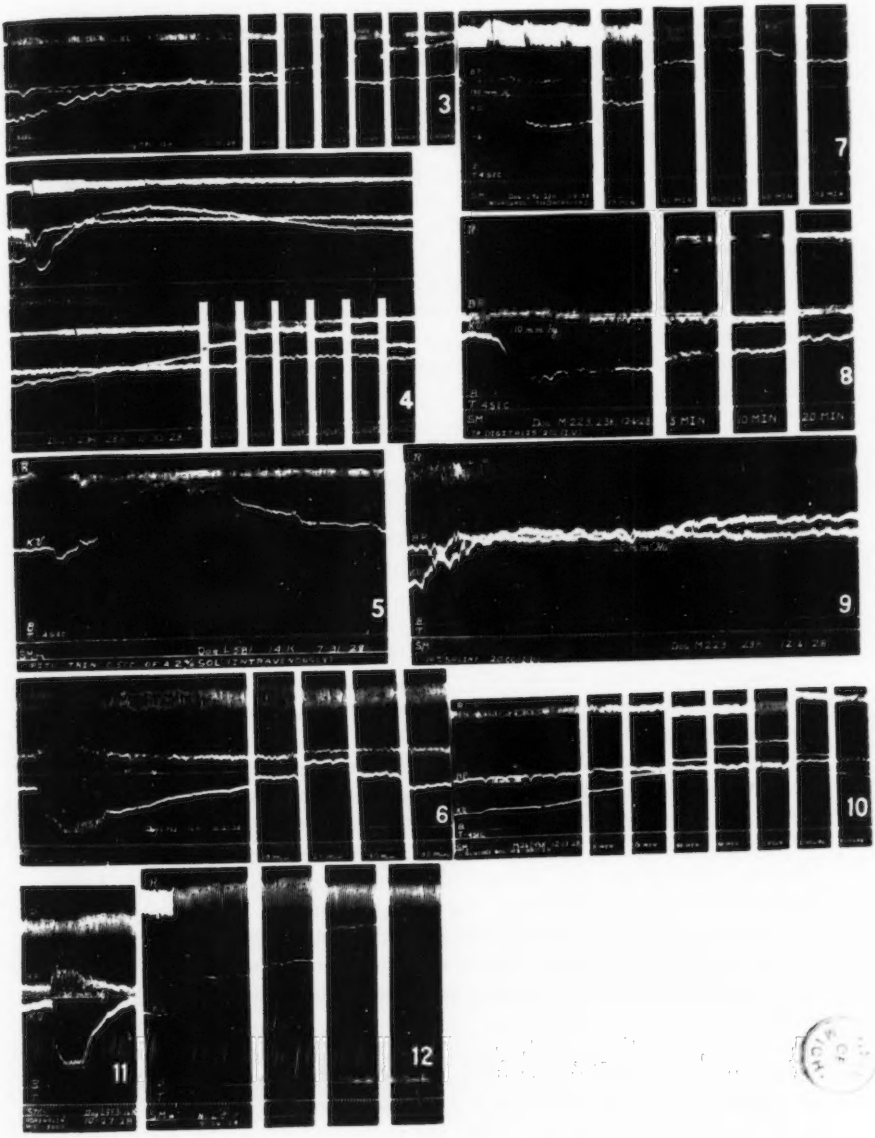
Fig. 8. Effect of a large dose of digitalis. There was prolonged decrease in renal volume which lasted twenty minutes.

Fig. 9. The effect of hypertonic solution of sodium chloride. Relatively small amounts produced marked increase in renal volume.

Fig. 10. Effect of 30 cc. of 10 per cent solution of glucose. There was pronounced and sustained increase in renal volume.

Fig. 11. Effect of a moderate dose of epinephrin on renal volume. The curve is typical.

Fig. 12. Effect of spinal anesthesia. The increase in renal volume was marked.



Figs. 3 to 12

caffeine sodio-benzoate. Both gave similar results, and the effect of varying doses on renal volume was chiefly a matter of degree. Immediately following the injection of caffeine, renal volume increased steadily, reaching its highest point one and a half to two hours later. This effect was maintained for approximately thirty minutes, after which the volume of the kidney slowly returned to normal. Blood pressure fell momentarily with the injection, but promptly recovered and maintained a constant level throughout the remainder of the experiment (fig. 3).

Theobromine sodio-salicylate. The effect of theobromine sodio-salicylate on renal volume was similar to that of caffeine. Varying amounts of from 5 to 15 grains were injected. The curve of renal volume was qualifatively the same with each injection. With larger doses of the drug there was more rapid and pronounced change, which is to be expected. The usual picture was a decided increase of renal volume, during or immediately after the injection; the volume returned to normal during the first ten or fifteen minutes. A steady increase then followed, reaching a maximum one and a quarter to one and a half hours after the administration of the drug. This maximal increase was maintained for a half-hour to an hour, after which the volume of the kidney slowly returned to normal. Blood pressure rose slightly with the injection, but fell to normal in ten minutes and remained almost constant during the remainder of the experiment.

Theophyllin-ethylene diamine. The drug was administered in small, moderate-sized, and large doses. The effects on renal volume were roughly similar to the effects of the other members of the caffeine group. On administration, there was a pronounced increase in renal volume before any change in blood pressure was manifested. A decided drop of the latter then occurred, followed by a corresponding and more pronounced decrease of renal volume. Both returned to normal in approximately one minute. Blood pressure then remained constant while the volume of the kidney steadily increased for several minutes. A secondary fall then occurred. This was gradual and not so marked as the former fall. With very large doses, however, this secondary fall may be absent. After eight to ten minutes a secondary increase began, reaching its highest point during the end of the first hour after injection. This increase was maintained for about forty minutes, after which a slow and gradual return toward normal began (fig. 4).

Pituitrin. The watery extract of the posterior lobe prepared in ampule form (pituitrin, surgical) was used. Varying amounts were administered. The smallest dose was 0.5 cc. of a 2 per cent solution; the largest was 0.5 cc. of the undiluted extract. With the minute doses only a slight initial decrease in renal volume occurred. This was followed at once by a pronounced increase, which lasted ten or fifteen minutes before the volume

returned to normal (fig. 5). The doses of moderate and larger size produced a correspondingly greater primary decrease of renal volume immediately after the injection. This decrease lasted several minutes before the volume returned to normal. A steady increase in volume then occurred, which reached its highest point twenty-five minutes after the injection, and then the volume gradually returned to normal. The entire process occupied thirty-five minutes. Blood pressure, as well as the pulse pressure, was increased immediately after administration of the drug, but fell to normal in two or three minutes and remained unchanged during the remainder of the experiment (fig. 6).

Merbaphen. The changes in renal volume produced by varying doses of merbaphen differed only in degree. The curves in all experiments were similar, and may be best described by a concrete example. A dog weighing 23 kgm. was given 0.7 cc. of merbaphen intravenously. Twenty seconds later, pronounced diminution of renal volume occurred, which lasted approximately ten minutes. A marked increase then followed, reaching a maximum during the forty-five-minute period, where it remained for an additional fifteen minutes. A slow and gradual return to normal then began (fig. 7). Blood pressure was not affected by the drug and remained relatively constant during the entire experiment.

Digitalis. The tincture of digitalis was used throughout. Small, moderate-sized, and large doses were administered. Doses of less than 5 minims failed to produce any visible change of renal volume in dogs weighing 14 to 15 kgm. Small doses produced slight reductions of renal volume, which were only momentary and equal amounts injected at intervals of two minutes produced slightly greater diminution of volume with each successive dose. With larger doses, the decrease of renal volume was more pronounced as well as prolonged, and often intervals of fifteen to twenty minutes were required before the volume returned to normal (fig. 8). Blood pressure was slightly increased with the larger doses, but returned to normal in two to three minutes and was unchanged during the remainder of the experiment.

Sodium chloride. Intravenous injections of isotonic solutions of sodium chloride, in amounts as small as 20 cc., produced a slight but definite increase in renal volume. Blood pressure was not affected. Hypertonic solutions of sodium chloride produced a rapid and more pronounced rise in renal volume. Twenty cubic centimeters of a 10 per cent solution caused not only a decided increase in renal volume but also a slight increase in blood pressure (fig. 9). The latter returned to normal in fifteen or twenty minutes, whereas approximately an hour was required for the volume of the kidney to become normal.

Glucose. Isotonic solutions of glucose in amounts up to 60 cc. may or may not cause an increase in renal volume. An increase was obtained in

about 50 per cent of the cases. When present, the increase was only slight, and the volume returned to normal within twenty minutes. Hypertonic (10 per cent) solutions of glucose in amounts of 20 to 30 cc. produced marked and prolonged increase in the volume of the kidney. The maximal increase was reached during the end of the first hour. The return to normal, however, required three hours (fig. 10).

Epinephrin. This drug was administered in amounts varying from 0.25 cc. of a 1:40,000 solution to 0.5 cc. of a 1:1000 solution. The effect on renal volume was invariably the same and the difference was chiefly a matter of degree. Immediately after the injection, curves typical of the effect of epinephrin on renal volume and blood pressure were obtained (fig. 11). In the morphinized animal, the curve of renal volume produced by epinephrin was decidedly more prolonged. The fall was somewhat slower; the recovery often required five to eight minutes, and in an exceptional case fifteen minutes.

Nitrites. Nitroglycerine was administered intravenously, and amyl nitrite was given by inhalation. The results on renal volume were similar. Both caused immediate diminution of renal volume, directly proportional to the amount administered.

Distilled water. The effect of distilled water on renal volume was remarkable. With the exception of epinephrin no substance used in this investigation produced the sudden and marked decrease effected by distilled water. This surprising result is dealt with in another paper (7).

The reflex response to various stimuli. During this investigation, it was observed that different external stimuli of unusual strength or quality often produced small but definite changes of renal volume. These changes were more pronounced in nervous dogs than in those with a quiet temperament. Usually, they were manifested by a momentary decrease in the volume of the kidney, but occasionally a slight increase occurred. As the animal became accustomed to his surroundings, these same stimuli had little, if any, effect and the response was often absent. This corroborates the results of Corbeille. The odor and sight of food produced a series of small, irregular waves which lasted until the stimulus was removed.

Spinal anesthesia. The effect of spinal anesthesia on renal volume may best be described by a concrete example. A dog weighing 15 kgm. was prepared for observation in the usual manner. Under local anesthesia, a needle was inserted into the spinal canal at the sixth dorsal vertebra, and a tracing of renal volume was made. Five cubic centimeters of a solution of 3 per cent procaine were slowly injected and subsequent tracings were taken at intervals of fifteen minutes. A pronounced increase in renal volume immediately followed the injection. This increase continued steadily for forty-five minutes, when a maximum was reached which lasted more than an hour (fig. 12). Below the level of the injection complete

paralysis occurred which lasted three or four hours. The animal, however, recovered completely and was in good condition the following day.

COMMENT. Before the results of this investigation can be established as physiologic facts, all sources of error must be eliminated.

The mechanical error produced by gross movements of the animal, such as struggling, or sudden changes of position, was avoided by using only well-trained animals for experimentation. Moreover, the respiratory tracing recorded simultaneously with renal volume shows graphically that the animal was quiet during the period of observation. The plethysmograph used in this study was sufficiently non-compressible to withstand all movements of the respiratory muscles, including the diaphragm. For this reason the curve of renal volume was practically unaffected by respiration, regardless of the type, rate, or amplitude. Movements of the stomach and spleen, which might have influenced changes in renal volume by tugging on the omentum, were avoided by careful selection of that portion used for covering the kidney and plethysmograph. This was done in such a way that when the kidney was restored to its normal position there was no tension whatever on the omentum between the stomach or spleen and the kidney.

With this method of plethysmography, necessarily a certain amount of exudate was formed in the instrument after its application to the kidney. This was most pronounced during the first twenty-four hours after operation, after which it gradually diminished. The amount present in the plethysmograph after an experiment two hours in duration usually was only a few drops, which is a negligible quantity. Occasionally, however, during observation of four or five hours, several cubic centimeters were formed. When this occurred, the results were discarded and the experiment repeated, using another animal if necessary. In a few instances, during long experiments, the few drops present were removed at the end of each hour. This permitted an accurate tracing of renal volume to be taken.

Finally, as a further precaution, when blood pressure was recorded simultaneously with renal volume, heparin was used in the connecting tubing rather than the usual anticoagulants, which have decided diuretic properties.

In this investigation it was observed that the changes in renal volume were, for the most part, similar to the results obtained by previous investigators on anesthetized animals. The prolonged increase in renal volume, following the administration of the common diuretics, was not accompanied by an appreciable change of blood pressure. The importance of recording blood pressure simultaneously with changes of volume in any organ is illustrated by the experiments with theophyllin-ethylene diamine, theobromine sodio-salicylate, digitalis and merbaphen. With the administration of theophyllin-ethylene diamine there occurs an im-

mediate and decided fall in renal volume accompanied by a marked decrease in blood pressure. On the other hand, theobromine sodio-salicylate promptly produces an increase in renal volume, and blood pressure is slightly elevated. Injections of merbaphen, however, do not affect blood pressure, whereas digitalis caused a slight increase, and both drugs invariably produce immediate diminutions of renal volume. Thus, although all four drugs are diuretics, their modes of action on the kidney and on blood pressure are obviously different.

The action of pituitrin, caffeine, and digitalis on renal volume closely paralleled the results obtained by previous investigators on anesthetized animals. However, with caffeine, the primary fall, of several minutes' duration, described by Phillips and Bradford, and others (2, 9, 10), was not obtained, and the fall in blood pressure was only slight. Digitalis invariably produced diminution of renal volume, which was directly proportional to the amount administered.

A decided decrease of renal volume was produced by the administration of amyl nitrite. Hargis and Mann observed a similar action of the drug on splenic volume, and Mattson obtained corresponding results on the liver. This, of course, is to be expected, for the nitrites apparently act primarily on peripheral arterioles rather than on those of the splanchnic area.

The greatest changes in renal volume were produced by the caffeine group of diuretics, especially with theophyllin-ethylene diamine. The action of merbaphen also was marked, but was not so prolonged as the caffeine diuretics. This parallels the degree of diuretic action manifested by these drugs clinically; the animals frequently voided large amounts of urine during the experiments. It is noteworthy that the influence of the diuretics mentioned is prolonged, and that often several hours are required for the influence to subside. Such prolonged observations could not easily have been made under anesthesia, without a probably serious depression of blood pressure, in which case a rise in renal volume would have been difficult to interpret.

The decided increase in renal volume following spinal anesthesia could well be explained on the basis of splanchnic paralysis.

In their plethysmographic study of the spleen, Hargis and Mann noted definite changes in volume accompanying various emotional states. Mattson observed similar variations in hepatic volume. Analogous changes occurred in renal volume. However, these were of less amplitude and of shorter duration. Characteristic alterations in renal volume also occurred with various external stimuli. These also were small as well as inconstant, unless new stimuli were introduced frequently. These facts indicate that there is a general vasomotor response in the splanchnic area during various emotional states. Moreover, it is probable that these changes in volume produced by various external stimuli also are accompanied by corresponding changes in the activity of the kidney.

SUMMARY

A method of plethysmographic study of changes in volume in the kidney has been described. This method permits observations to be made easily on virtually intact dogs. Essentially, this method is an adaptation of the collodion plethysmograph of Hargis.

Studies in relation to renal volume were made with the following: caffeine, theobromine sodio-salicylate, theophyllin-ethylene diamine, pituitrin, digitalis, merbaphen, the nitrites, epinephrin, spinal anesthesia, and distilled water.

In many experiments simultaneous tracings of carotid blood pressure were made under local anesthesia.

The changes in volume agreed for the most part with the results obtained by other investigators on anesthetized animals. However, with the diuretics these changes often were decidedly more marked and more prolonged, frequently lasting for hours.

As observed by Hargis and Mann in their studies on splenic volume, the kidney showed analogous changes in response to various external stimuli, such as a sudden or unusual noise, or the odor of food.

A remarkable action of distilled water on renal volume was observed. The administration of 0.5 to 1.0 cc. for each kilogram of body weight produced a temporary but pronounced decrease in volume, without change in blood pressure.

The plethysmograph described is recommended for use in acute experiments as well as in the prolonged type reported in this paper.

BIBLIOGRAPHY

(1) Corbeille, C. Changes in the volume of the kidney due to acoustic stimulation. (Unpublished data.)
(2) Fletcher, W. M., V. E. Henderson and O. Loewi. Arch. f. exper. Path. u. Pharm., 1905, liii, 15.
(3) Hargis, E. H. Journ. Lab. Clin. Med., 1925–1926, xi, 664.
(4) Hargis, E. H. and F. C. Mann. This Journal, 1925–1926, lxxv, 180.
(5) Mattson, H. This Journal, 1929, xc, 146.
(6) Phillips, C. D. F. and J. R. Bradford. Journ. Physiol., 1887, viii, 117.
(7) Reid, W. L. This Journal, 1929, xc, 168.
(8) Schäfer, E. A. and P. T. Herring. Phil. Trans. Roy. Soc. London, 1908, B, cxcix, 1.
(9) Starling, E. H. Journ. Physiol., 1899, xxiv, 317.
(10) Thompson, W. H. Journ. Physiol., 1899–1900, xxv, 487.

THE EFFECT OF INTRAVENOUS INJECTIONS OF DISTILLED WATER ON THE KIDNEY

W. L. REID

From the Division of Experimental Surgery and Pathology, The Mayo Foundation, Rochester, Minnesota

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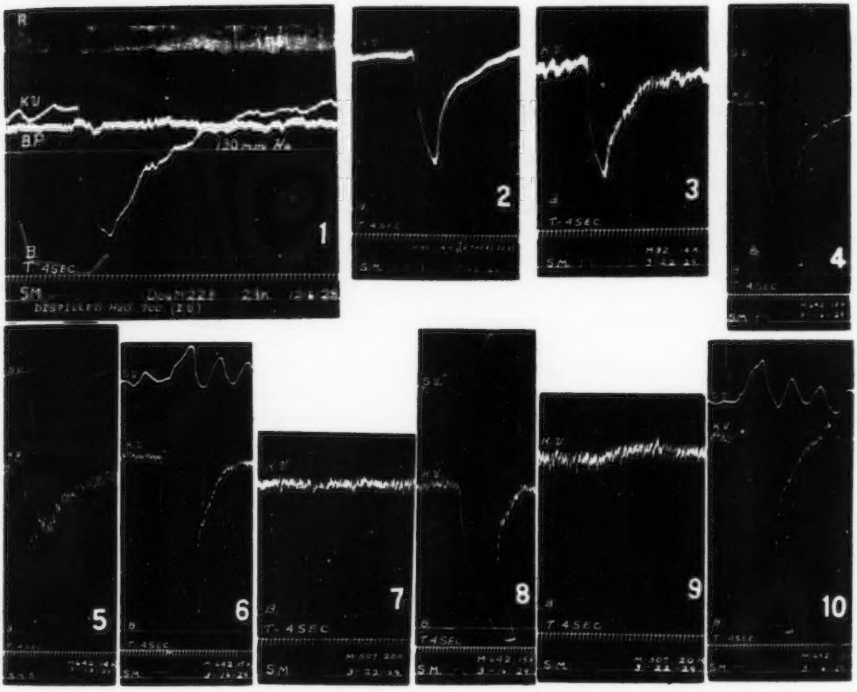
In the course of a recent investigation of changes in renal volume in the virtually intact animal, it was observed that intravenous injections of distilled water in amounts of 0.5 to 1.5 cc. for each kilogram of body weight produced marked decrease in the volume of the kidney. This decrease was rapid but of short duration; the volume of the kidney returned to normal in one to two minutes. The volume curve was similar to that produced by intravenous injection of a moderate amount of epinephrin. However, blood pressure remained unaffected (fig. 1). In view of this remarkable action of so small an amount of distilled water on the volume of the kidney, an analysis of the factors concerned with its production was begun. The method used in this investigation has been described in detail in another paper (1).

There were three possible explanations for the transitory decrease of renal volume. The distilled water could have produced dilution of the blood which might cause specific changes due to 1, central stimulation or 2, peripheral stimulation, and 3, a similar result could have occurred from changes in the blood itself, produced in that portion flowing past the point of the needle during the injection.

It was first determined that the striking decrease in renal volume was due to the osmotic effect of the distilled water, because the addition of sodium chloride or glucose to the water invariably abolished the response. It also was observed that anesthesia did not abolish the response, for a similar decrease was obtained in the etherized animal (fig. 2). The action appeared specific for the kidney, for blood pressure was not affected and simultaneous records of volume of the spleen and of the kidney did not show decrease in volume of the kidney at any time (fig. 3). The mechanism producing this decrease in volume was found to be a peripheral one, because similar results were obtained on the denervated kidney when distilled water was injected (figs. 4 and 5). These various observations indicated that injections of distilled water produced specific vasoconstriction of the renal vessels due to peripheral stimulation. Accordingly, the



Figs. 1 to 10

Fig. 1. The effect of intravenous injection of distilled water on renal volume. Marked decrease in volume resulted from the administration of 0.3 cc. for each kilogram of body weight (7 cc. intravenously). Blood pressure was not affected. *R*, respiration, *K.V.*, volume of kidney; *B.P.*, blood pressure; *B*, base line; *T*, time in intervals of four seconds.

Fig. 2. The effect of distilled water on renal volume in the etherized animal. The usual characteristic decrease of renal volume occurred. Twenty cubic centimeters were injected. *K.V.*, volume of kidney; *B*, base line; *T*, time in intervals of four seconds.

Fig. 3. The effect of distilled water on the denervated kidney. The usual characteristic decrease of renal volume resulted from the injection. Twenty cubic centimeters were injected. *K.V.*, volume of kidney; *B*, base line; *T*, time in intervals of four seconds.

Fig. 4. Twenty cubic centimeters of distilled water produced a marked decrease in renal volume and a slight increase in the volume of the spleen. *S.V.*, volume of spleen; *K.V.*, volume of kidney; *B*, base line; *T*, time in intervals of four seconds.

Fig. 5. The characteristic changes in volume in both the spleen and kidney were obtained with the injection of 0.005 cc. of 1:1000 solution of epinephrin. *S.V.*,

volume of spleen; *K.V.*, volume of kidney; *B*, base line; *T*, time in intervals of four seconds.

Fig. 6. The injection of 5 cc. of normal blood, laked with 5 cc. of distilled water to which was then added 10 cc. of 2 per cent solution of sodium chloride, produced a marked decrease of renal volume and a slight increase in the volume of the spleen. *S.V.*, volume of spleen; *K.V.*, volume of kidney; *B*, base line; *T*, time in intervals of four seconds.

Fig. 7. Renal volume was not affected by the injection of a mixture of 10 cc. of plasma and 10 cc. of distilled water to which 5 cc. of 2 per cent solution of sodium chloride were then added. *K.V.*, volume of kidney; *B*, base line; *T*, time in intervals of four seconds.

Fig. 8. The injection of 5 cc. of red blood cells laked with 5 cc. of distilled water, to which were then added 10 cc. of 2 per cent solution of sodium chloride, resulted in a marked decrease of renal volume and a slight increase in the volume of the spleen. *S.V.*, volume of spleen; *K.V.*, volume of kidney; *B*, base line; *T*, time in intervals of four seconds.

Fig. 9. The administration of 5 cc. of intact red blood cells in 15 cc. of physiologic solution of sodium chloride produced a slight but definite increase in renal volume. *K.V.*, volume of kidney; *B*, base line; *T*, time in intervals of four seconds.

Fig. 10. The injection of 5 cc. of red blood cells, laked with ether, then suspended in 5 cc. of physiologic solution of sodium chloride, produced the greatest decrease in renal volume of the series of experiments. *S.V.*, volume of spleen; *K.V.*, volume of kidney; *B*, base line; *T*, time in intervals of four seconds.

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following series of experiments was done: 1. Blood was taken from a normal dog and laked by the addition of an equal amount of distilled water. Sufficient amounts of 2 per cent solution of sodium chloride were then added to make the laked blood definitely hypertonic. Injections of the resulting solution produced the same type of decrease in renal volume as that produced by injection of distilled water (fig. 6). 2. Specimens of normal blood were centrifuged and the plasma and cells carefully separated. Distilled water was added to both and the two solutions were rendered hypertonic with a given quantity of 2 per cent solution of sodium chloride. With injections of the plasma a decrease in renal volume did not occur, whereas injections of the blood cells produced a marked decrease (figs. 7 and 8). 3. Finally, blood from a normal dog was laked with ether and suspended in physiologic solution of sodium chloride. Injections of this solution caused a pronounced decrease of renal volume (fig. 9). 4. Repeated administration of washed blood cells invariably resulted in a slight increase in renal volume with each injection (fig. 10).

The implications from the results of these experiments are quite clear. They show conclusively that the decrease in volume was due to some substance produced by distilled water injuring the erythrocytes. It is evident, therefore, that when the erythrocytes are injured or destroyed a substance

is produced which causes specific vasoconstriction of the renal vessels, resulting in marked transitory decrease of renal volume.

It is interesting to speculate on the nature of this substance, and also on its possible relation and application to clinical medicine, but further studies must be made before it can be identified.

BIBLIOGRAPHY

(1) Reid, W. L. This Journal, 1929, xc, 157.

A QUANTITATIVE DETERMINATION OF THE CHANGES IN HEMOGLOBIN CONCENTRATION, VOLUME OF RED CELLS, AND BASOPHILIA IN THE BLOOD OF RABBIT FETUSES AT VARIOUS STAGES DURING THE LAST THIRD OF PREGNANCY

LOUIS D. ZEIDBERG

From the Department of Anatomy, University of Rochester, School of Medicine and Dentistry

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In the course of recent observations in this laboratory on the changes in the blood of rabbit fetuses at various stages of pregnancy, it has been noted that in the case of metabolites such as glucose (1) and amino-acids (2), the concentration in fetal and maternal blood may not be equal. Indeed, in the case of glucose it has been found that as term is approached there is a gradual increase in the reducing substance present in fetal blood so that the blood sugar of the fetus at term is about three times as great as at the beginning of the last third of the gestation period.

In view of the prominence of oxygen in the utilization of carbohydrate by the animal, and the accuracy with which the oxygen carrier of the blood, hemoglobin, can be measured, it would seem especially interesting to determine the quantity of hemoglobin present per unit volume of fetal blood at various stages of pregnancy. Indeed, if it can be shown that there is a striking increase in the hemoglobin concentration of fetal blood during the last third of pregnancy, which would thus be coincident with a marked rise in the blood sugar of the fetus, there would be at hand evidence of the quantitative relation during a considerable part of pregnancy of two outstanding factors of the "milieu interieur" of the fetus, especially with regard to the utilization of carbohydrate.

Moreover, in so far as the amount of hemoglobin present in fetal blood enters into the calculation of the oxygen and carbonic acid exchange between mother and fetus across the placental boundary, the present observations would provide essential quantitative data; and the activity of the placenta in the rôle of the "pulmo uterinus" as Harvey referred to it, might be somewhat more clearly defined.

Historical. Previous attempts to measure the hemoglobin concentration of fetal blood at various stages of pregnancy have been rather limited. In 1928, Nicholas (3) and Bosworth in observations on rat fetuses found that there was an increase in hemoglobin from about 5 grams per 100 cc.

of blood on the 12th day of pregnancy to about 9 grams at term, the 22nd day. Apparently considerable technical difficulties were encountered.

No previous observations have been found which deal with changes in the volume per cent of the fetal red blood cells based on hematocrit determinations at various stages of pregnancy.

A full description of the histological changes in the red cells in certain species from the time of earliest differentiation is available in the well known studies of Doan (4), Cunningham and Sabin. Of immediate interest in connection with the present investigation is the relation between basophilia and the appearance of hemoglobin in the cytoplasm, as shown by Sabin (5) in a diagram of the maturation of red blood cells (p. 225, chart 2). A paper of Key (6) dealing with polychromatophilia is also of interest. Despite the previous detailed histological studies, however, it would seem that at present the fetal blood has not been observed at close intervals during the latter part of pregnancy with a view to the quantitative determination of the decrease in basophilic red cells.

It is evident that in previous observations the measurement of the hemoglobin concentration of fetal blood at various stages of pregnancy has been limited, and that hematocrit determinations of cell volume are apparently untried. In no case have the colorimetric estimations of hemoglobin been systematically checked by morphological findings in the same animals. The present experiments, therefore, represent a correlated study of the changes in the fetal red cells as revealed by quantitative determination of hemoglobin content, of the cell volume, and of the percentage of basophilic cells at various stages during the last third of pregnancy.

METHOD AND MATERIAL. The general plan in the present investigation has been to determine the hemoglobin content of the blood by Kennedy's (7) method, using a Du Boscq colorimeter. This colorimetric estimate is checked by histological examination of the red cells in smears prepared by Jenner's modified Romanowsky stain, and the proportion of basophilic red cells is counted. Further verification of the hemoglobin concentration is obtained by hematocrit determinations giving the volume of red cells per unit volume of blood. In all cases the blood of the mother is studied, as well as of the fetuses.

In a typical experiment the procedure was as follows. Blood was obtained from the marginal ear vein of the mother, and amytal was then given, usually by stomach tube, in dosage of 0.07 gram per kilo body weight. In some cases 0.05 gram per kilo was given subcutaneously. When the animal was anesthetized, generally within an hour, the abdomen was opened by an incision in the midline, 5 cm. in length, and usually not more than 1 cc. of blood was lost. Through this opening the position of the fetus was determined by palpation. An opening was made in the thin uterine wall with little bleeding by spreading the blunt ends of a

Halsted clamp in order to divide the tissues. The fetus was then extruded, enclosed in the membranes, which were immediately punctured, and after the escape of the amniotic fluid the umbilical cord was ligated to prevent the loss of blood. The placenta was not removed, but was left attached in the uterus. The abdominal wall was closed with a clamp and blood was taken from this embryo before the next one was removed from the uterus. The fetus was thoroughly dried and blood was obtained by cutting the jugulars. Care was taken to leave the trachea and esophagus intact in order to avoid possible contamination of the blood by fluids which might escape from these passages. As the blood dropped from the vessels it was caught on a clean watch glass and immediately taken up in the measuring pipette.

In the determination of hemoglobin 0.1 cc. of blood was measured with an accurately calibrated pipette, and then collected in a 10-cc. volumetric flask. The pipette was rinsed and the blood sample diluted to the 10 cc. mark with 0.4 per cent ammonium hydroxide solution. The colorimetric reading was taken within an hour after laking the blood.

The method for the estimation of hemoglobin by the use of light filters as described by Kennedy (1926) was used. Through the courtesy of Doctor Kennedy it was possible in the present observations to use the identical light filters employed by him and standardized by means of oxygen capacity determinations of hemoglobin. In this method a neutral gray light filter of known density was placed on one cup holder of a Du Boscq colorimeter and the diluted blood was put in a cup on the other side. A light filter, Wratten no. 74, monochromatic green, was placed in the eye piece of the colorimeter and the match point determined. The reading obtained on the scale of the colorimeter was applied to a formula $C = A \cdot \frac{D}{h}$, where C is the concentration of hemoglobin in grams per 100 cc. of blood; A, a constant, is the absorption ratio; D is the density of the neutral gray light filter; and h is the reading on the colorimeter scale. The value 14.8 was used for A. Kennedy found the error of the method to be about 3 per cent. It was well adapted to the rapid handling of an entire litter of fetuses.

Observations were made on 87 fetuses of 19 rabbits during the last third of gestation, as listed in the table. Duplicate determinations were regularly taken in the case of maternal blood, and in numerous instances in the case of fetuses.

A rough estimation of the hemoglobin content of the blood was obtained by histological examination of smears of fetal blood stained with Jenner's mixture. Usually smears were made from 3 fetuses of a litter and 1000 cells were counted to determine the percentage of basophilic red cells present. Nine pregnant rabbits were observed. (Table.)

The measurement of the volume per cent of the red cells was made

with the bulb pipette as described by Van Allen (8). A unit quantity of blood, usually 0.02 cc., was drawn into the pipette as soon as possible after bleeding the fetus, and mixed at once with an arbitrary amount of 1.4 per cent sodium oxalate solution. The ends of the pipette were sealed off with a rubber band. The pipette was placed in a centrifuge cup and spun at the rate of 2400 revolutions per minute for 35 minutes. The height of the column of red cells was read on the graduated scale of the tube directly in terms of the volume per cent of the red blood corpuscles. The same centrifuge with a constant load was used throughout.

Observations on the red cell volume are based upon 49 fetuses obtained from 12 rabbits at close intervals during the last third of gestation, as listed in the table. The maternal blood was studied similarly and determinations made in duplicate.

Regarding the animals used in the present experiments, it may be noted that the series of rabbits was sacrificed at close intervals during the last third of the gestation period, i.e., between the 22nd and 32nd day of pregnancy. The stage of pregnancy is estimated from the time of mating, and may be calculated with surprising accuracy in the rabbit, since ovulation occurs about 10 hours after coitus (9). The animals were apparently in good health and the mothers were for the most part reared in the laboratory. They were provided abundantly with a standard diet which consisted of cabbage, alfalfa, oats, salt and water. The rabbits were usually isolated in individual cages throughout pregnancy. The visceral organs were examined at autopsy for macroscopic lesions.

Observations. In the present observations on the blood of rabbit fetuses there is evidence of a striking increase in the hemoglobin concentration per unit volume of blood during the last third of pregnancy. In the determination of hemoglobin based upon colorimetric estimations, the microscopical examination of histological preparations, and the hematocrit readings of red cell volume, there is uniform agreement in pointing to a marked increase in the quantity of hemoglobin per unit volume of fetal blood during the last third of gestation.

The changes which have been noted in the coloring power of the fetal blood can be expressed in terms of grams of hemoglobin per unit volume of blood by means of the colorimetric method of Kennedy. Thus it has been found that in 22 day fetuses which weigh about 5 grams the hemoglobin averages about 8 grams per 100 cc. of blood, while at term (32 days) in fetuses averaging about 48 grams in weight the fetal blood averages about 12 grams of hemoglobin per 100 cc. This is an increase in hemoglobin of about 50 per cent.

The observation of so large an increase in the hemoglobin of fetal blood is further checked by the findings in the blood of fetuses at various stages intermediate between the 22nd and 32nd day of pregnancy. As may

TABLE 1

MATERNAL					FETAL						
Rabbit number	Day of gestation	Weight	Hemoglobin per 100 cc.	R.B.C. volume	Average weight of fetuses	Fetus number	Hemoglobin per 100 cc.	Average hemoglobin per 100 cc.	R.B.C. volume	R.B.C. average volume	Basophilic R.B.C.
		kgm.	*grams*	*per cent*	*grams*		*grams*	*grams*	*per cent*	*per cent*	*per cent*
75	21		11.0	37.0	4	1	6.6	6.6			
101	22	3.5	12.2	36.0		1	6.3		26.0		
						2	6.9		27.0		
						3	9.4				
209		2.7	11.8	34.0		1	8.6				
						2	8.4		27.0		
						3	8.3		27.0		
233		3.7	11.5	33.0		1	8.6				
						2	7.1				
						3	6.5				
						4	8.4		31.0		
						5	7.3				
						6	8.9				
						7	9.0				
						8	8.0				
						9	9.0				
						10			28.0		
					5			8.0	28.0	28.0	28
83	23	3.5	12.5	37.0		1	8.6		32.0		
						2	8.0				
						3	9.9	8.8		32.0	26
227	24	4.55	11.2	34.0		1	9.5		33.0		
						2	8.6		32.0		
						3	8.8		36.0		
						4	7.7				
						5	7.5				
						6	7.9		31.0		
						7	7.9		34.0		
						8	7.9		36.0		
						9	8.6				
					9	10		8.4		33.0	
269		2.9	10.3	29.0		1	9.7				
						2	8.4		32.0		
					11	3	9.6		32.0	32.0	
						4	8.6	9.2			
						5			32.0		
						6	9.6				

TABLE 1—*Continued*

MATERNAL					FETAL						
Rabbit number	Day of gestation	Weight	Hemoglobin per 100 cc.	R.B.C. volume	Average weight of fetuses	Fetus number	Hemoglobin per 100 cc.	Average hemoglobin per 100 cc.	R.B.C. volume	R.B.C. average volume	Basophilic R.B.C.
		kgm.	*grams*	*per cent*	*grams*		*grams*	*grams*	*per cent*	*per cent*	*per cent*
226	25	3.5	11.5	34.0		1	10.6		36.0		
						2	10.1		35.0		
						3	9.8		38.0		
						4	11.4		40.0		
						5	9.9		35.0		
						6	9.9		36.0		
						7	9.6		32.0		
						8	9.0		34.0		
					16	9	10.7	10.1		36.0	20
68	26	3.3	10.7	31.0		1	11.0				
						2	10.9				
					20	3	10.7	10.9			16
217	27	3.7	11.0	38.0		1	11.7		38.0		
						2	11.1				
						3			36.0		
						4			38.0		
229		4.3		26.0		5			44.0		
						6			42.0		
						7			40.0		
					23	9		11.4	37.0	39.0	12
230	28	4.2		29.0		1			38.0		
						2			44.0		
						3			39.0		
						4			44.0		
						5			44.0		
						6	11.8		40.0		
						7	11.9	11.9	40.0	41.0	8
203	29	3.5	10.8	34.0		1	11.2				
						2	12.1				
						3	13.1				
215		3.7	12.1	32.0		1	12.0				
						2	11.8				
						3	12.2				
						4	11.3				
					39	5	11.8	12.0			4

TABLE 1—*Concluded*

MATERNAL					FETAL						
Rabbit number	Day of gestation	Weight	Hemoglobin per 100 cc.	R.B.C. volume	Average weight of fetuses	Fetus number	Hemoglobin per 100 cc.	Average hemoglobin per 100 cc.	R.B.C. volume	R.B.C. average volume	Basophilic R.B.C.
		kgm.	*grams*	*per cent*	*grams*		*grams*	*grams*	*per cent*	*per cent*	*per cent*
84	30	3.9	14.7			1	11.5				
						2	11.2				
X		3.8				1	12.9		41.0		
						2	11.8		39.0		
						3	13.8		42.0		
						4	11.6		44.0		
					35	5		12.1	39.0	41.0	4
7	31	3.2	12.0	38.0		1	11.7		42.0		
						2	12.4		43.0		
						3	11.6		43.0		
						4	10.9		42.0		
						5	11.9				
					40	6		11.7	42.0	42.0	
74	32	3.9	14.0	39.0		1	12.8				
211		5.9	11.3	34.0		1	11.8				
					48	2	12.3	12.3			4

be seen in the table, the hemoglobin concentration per unit volume of blood tends to increase gradually as term is approached. This may be more clearly expressed perhaps, as in figure 1, by a curve drawn through the arithmetic mean of the observed values at various stages of gestation.

In control determinations on the maternal blood it is found that the hemoglobin may range from 10.3 grams to 14.7 grams per 100 cc. of blood. (Table.) However, in the case of the maternal blood it is not possible in this series to establish a clear-cut correlation between the hemoglobin concentration and the stage of pregnancy, such as has been demonstrated in the case of fetal blood.

Evidence of marked histological changes in the hemoglobin bearing cells of fetal blood during the last third of pregnancy is found in smear preparations. A quantitative estimate of the changes has been attempted by enumeration of the percentage of basophilic red cells in smears obtained at various stages of pregnancy, and stained with Jenner's modified Romanowsky stain. Thus it has been found that in 22 day embryos which average about 5 grams in weight there are about 28 per cent basophilic red cells in the smears, while in contrast in 32 day fetuses, which average

about 48 grams in weight, there are about 4 per cent basophilic red corpuscles in the smears.

Observations on smears of fetal blood obtained at stages intermediate between the 22nd and 32nd day of pregnancy likewise show that there is a gradual decrease in the percentage of basophilic red cells as term is approached. (Table.)

In the maternal blood, no changes of this sort were found in histological preparations.

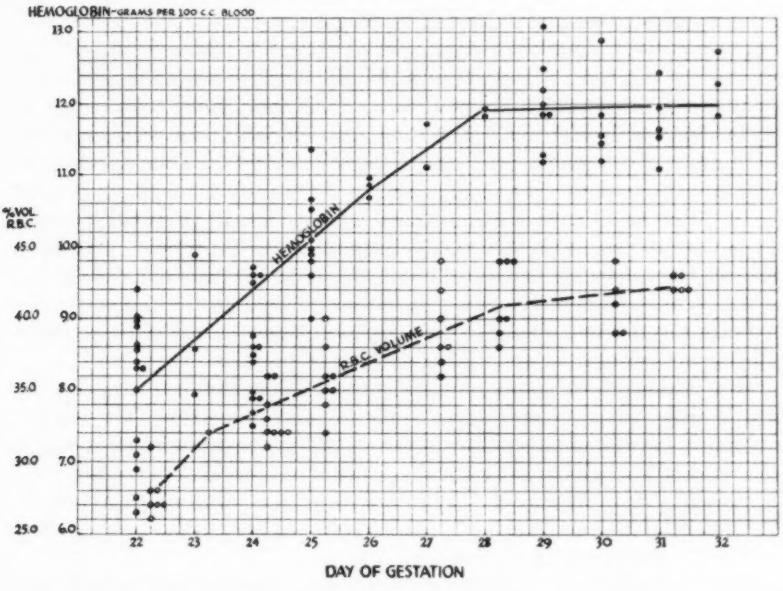

Fig. 1. The solid dots represent individual determinations of hemoglobin. The circles represent observations of the per cent volume of the R.B.C. The curves are drawn through the arithmetic mean of all the observed values at each day of gestation.

In addition to the increase in hemoglobin as determined by colorimetric measurement, and the decrease in basophilic red cells as seen in smears, a third line of evidence has been obtained regarding the nature of the changes in the fetal red cells, namely, the variation in the volume of red cells per unit volume of blood at various stages of pregnancy. Thus it has been found that in 22-day fetuses weighing about 5 grams the red cell volume per cent averages about 28, while in 31-day fetuses weighing about 40 grams the red cell volume per cent averages 42. This is an increase in volume of red cells of about 50 per cent. Observations on fetal blood obtained

at stages of pregnancy intermediate between the 22nd and 31st day show that there is a gradual increase in volume of red cells per unit volume of blood as term is approached. (Table.) This is summarized in a curve (fig. 1) which is drawn through the arithmetic mean of the observed values arranged according to the stage of pregnancy.

In control observations on the maternal blood, variations were observed in the volume per cent of the red cells, which ranged from 26 to 39 volumes per cent in different animals. However, the significance of the variations in the maternal blood is still difficult to determine after plotting the observed values according to the stage of pregnancy.

Discussion. From the foregoing observations it becomes evident that during the last third of gestation in the rabbit, there are striking changes in the fetal blood, which are marked by an increase in the coloring power and volume of the red cells per unit volume of blood, and a decrease in the percentage of basophilic red corpuscles seen in smears. Thus colorimetric determinations show that there are about 8 grams of hemoglobin per 100 cc. of blood at 22 days, while at term there are about 12 grams of hemoglobin per 100 cc. of blood, or an increase of about 50 per cent. Hematocrit readings show an increase of about the same magnitude in the volume of red cells. Thus in the blood of fetuses at 22 days the red corpuscles average about 28 volumes per cent, while at term the red cells are about 42 volumes per cent, or an increase of about 50 per cent.

It is interesting to note that the increase in hemoglobin is paralleled by an increase in the volume of red cells in such proportion that a given volume of red cells obtained from fetuses at 22 days contains about the same quantity of hemoglobin as an equal volume of red corpuscles obtained from fetuses at term. As may be seen in the graph, the curve which represents the hemoglobin concentration is remarkably similar to the curve which is plotted for the volume of red cells.

Nevertheless there are marked structural changes in the red cells of the fetus during the last third of pregnancy. Thus in histological preparations of blood of 22-day fetuses there are many normoblasts, and basophilic red cells make up about one-fourth of the total number of red corpuscles (fig. 2). On the other hand in fetuses at term there are only about 4 per cent basophilic red cells.

The marked erythropoietic activity observed in 22-day fetuses is of interest also in connection with the studies of Doan (4), Cunningham and Sabin on the nature of the stimuli which are operative in hematopoiesis. These authors point out that the stimuli upon which the initiation of the erythropoietic origin and maturation depend are probably a low oxygen tension in the portion of the marrow where they are developing, dependent upon the nature of the vascular bed in those areas and upon a hypothetical chemical substance in the nature of a gel which has been observed to

constantly accompany the active formation of erythrocytes. Indeed, the direct experimental production of erythropoiesis in animals kept under reduced oxygen tension is described by Dallwig (10), Kolls, and Loevenhart.

In a few observations on the average diameter of red cells which show no basophilic staining, it would seem that there tends to be a decrease in diameter during the last third of pregnancy which ranges from a diameter of more than 8 microns in fetuses at 22 days to a diameter of less than 7 microns in erythrocytes of fetuses at term. This impression is based upon the examination of smears obtained at various stages of pregnancy, and about 50 cells were measured in each preparation under an oil immersion lens, using an ocular micrometer.

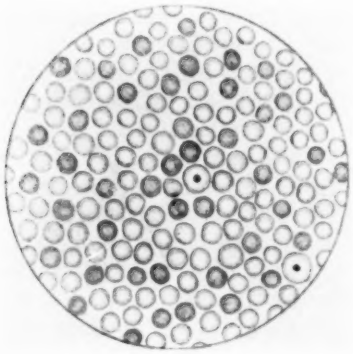

Fig. 2. Typical microscopic field of a blood smear of a 22-day rabbit fetus, showing about 28 per cent basophilic R.B.C., and two nucleated cells.

The rapidity of the changes in the fetal blood is illustrated in the graph (fig. 1). It is apparent that the marked increase in hemoglobin and red cells, along with the gradual disappearance of the immature red corpuscles occurs until about the 28th or 29th day of pregnancy at which time apparently a level is approached which remains fairly constant until parturition at about 32 days.

It is evident that the increase in hemoglobin concentration in the rabbit embryo is coincident with the approach of the day when the embryo will be extruded suddenly into an external environment which is in great contrast with the watery surroundings of the fetus in utero. In intra-uterine life, the embryo obviously exerts little or no muscular activity in such fundamental processes as respiration, nutrition, and body temperature regulation. This is in striking contrast, of course, with the active movements of the newborn in breathing, suckling, and keeping warm.

The onset of increased muscular activity in the newborn is also marked by a high hemoglobin concentration in the blood, which is thus in contrast with the relatively low hemoglobin of embryos at 22 days.

In this connection perhaps one may take into consideration the recent observations of Hall (11) and Gray dealing with variations in hemoglobin concentration in certain vertebrates which remain in an aquatic habitat throughout life. These authors point out that the hemoglobin concentration of marine fishes is perhaps an index of their physiological activity. The more active species appear to have the highest hemoglobin concentration and the less active species have the lowest concentration, according to a study of 15 species of teleosts. An active species, like the bull's-eye mackerel, which is constantly moving rapidly through the water has a hemoglobin concentration probably 3 times as great as a sluggish species, such as the toadfish, which tends to lie in wait for animals that may come by chance within its reach. In a further study on the influence of varying oxygen tensions upon the rate of oxygen consumption, Hall (12) points out that a fish having a higher concentration of hemoglobin is apparently able to expend its energy at a faster rate and conversely, one with a low concentration carries on its metabolism at a lower rate.

Finally, if the changes in concentration of fetal hemoglobin are considered in the light of the functions of hemoglobin in the animal, it might be inferred that there are far-reaching physiological changes in the fetal organism during the last third of pregnancy. These changes, it would seem, must involve the transport of oxygen, the transport of carbonic acid, and the regulation of alkalinity. Thus the difference in concentration of hemoglobin on the fetal and maternal sides of the placental boundary, for instance in 22-day fetuses, must raise the question as to the nature of the regulatory mechanism involved in the respiratory processes of the embryo. It would seem to be evident, therefore, that the epithelial membrane which constitutes the placental barrier between the maternal and fetal organisms must be taken into account in any attempt to elucidate the processes which are concerned in compensating for the striking differences between maternal and fetal blood at various stages of pregnancy.

SUMMARY

In the blood of rabbit fetuses during the last third of pregnancy, i.e., 22nd to 32nd day, it is found that:

1. The hemoglobin concentration increases from an average of about 8 grams per 100 cc. of blood at 22 days to about 12 grams per 100 cc. of blood at term, or roughly an increase of 50 per cent.

2. The volume of red corpuscles increases from about 28 volumes per cent at 22 days to about 42 volumes per cent at term, or roughly an increase of 50 per cent.

3. The percentage of basophilic red cells in histological preparations decreases from about 28 per cent at 22 days to about 4 per cent at term.

My thanks are extended to Dr. F. F. Snyder who aided and encouraged this work.

BIBLIOGRAPHY

(1) Synder and Hoskins: Anat. Record, 1928, xxxviii, 28.
(2) Snyder and Hoskins: Unpublished observations.
(3) Nicholas and Bosworth: This Journal, 1928, lxxxiii, 499.
(4) Doan, Cunningham and Sabin: Contrib. to Embryol., 1925, xvi, 163.
(5) Sabin: Physiol. Reviews, 1928, viii, 191.
(6) Key: Arch. Int. Med., 1921, xxviii, 511.
(7) Kennedy: This Journal, 1926, lxxviii, 56.
(8) Van Allen: Journ. Lab. Clin. Med., 1925, x, 1027.
(9) Snyder: Anat. Record, 1926, xxxii, 242.
(10) Dallwig, Kolls and Loevenhart: This Journal, 1915, xxxix, 77.
(11) Hall and Gray: Journ. Biol. Chem., 1929, lxxxi, 589.
(12) Hall: This Journal, 1929, lxxxviii, 212.

THE EFFECT OF FASTING ON THE KNEE JERK, STANDING STEADINESS, AND THE SALIVARY REFLEX[1]

MARY A. M. LEE

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The effect of a five day fast upon the knee jerk was first observed by us in the course of an investigation concerning the reliability and significance of knee jerk measurements. During the months of November and December, 1922, the two subjects were tested daily under standardized conditions, both subjects underwent a five day fast. In each case the knee jerk almost entirely disappeared in the course of the five days. Our long control periods had established the limits within which the knee jerk of each subject may be said normally to fluctuate from day to day and the depression which occurred in fasting was definitely outside these limits. This effect was in contrast to the absence of consistent change in the amplitude of the knee jerk as a result of experimental insomnia, two periods of which subject K also underwent during these months (1). To determine whether this change in the patellar reflex in fasting, so marked in these two subjects, was true for others as well, the following experiment was undertaken. Chronologically it falls into three parts. The first consists in the two subjects described above and four more subjects who were tested at that time during a five day fast but with shorter control periods. The second experiment was carried out the following year at our request by Dr. A. H. Steinhaus on three subjects, using the same apparatus and technique. In the fall of 1927, six medical students undergoing the five day fast as a required part of their work in physiology consented to serve as subjects for experiment 3. At this time it was thought desirable to find out if other reflexes were affected. The Romberg test, or standing steadiness, had proved sharply sensitive to the condition of experimental insomnia in our hands, and was therefore included. A salivary reflex was selected also, because of its availability for quantitative measurement, because no gross muscular component was involved in it, and finally because there were already some data published regarding it in this little investigated field.

[1] Studies from the Institute for Juvenile Research, Chicago; Herman M. Adler, M.D., Director. Series B, No. 141.

This work was done at the Hull Physiological Laboratory of the University of Chicago. I wish to express my gratitude to Dr. A. J. Carlson for his interest and valuable advice.

Kleitman and Crisler in 1927 reported the loss of a conditioned salivary reflex in dogs as a result of starvation (2). That this was due to a change in the central nervous system Kleitman demonstrated later by showing that salivation resulting from pilocarpine injection is somewhat decreased in starvation but not lost (3). He concluded that the difference in extent of effect was due to the fact that here there was involved only the secreting mechanism and no central component (since a conditioned reflex cannot be built up using pilocarpine as a stimulus).

In human beings few adequate studies of the effect of fasting on physiological activities have been made. The fasts of Levanzin or Succi reported by Langfeld and Luciani respectively (4), (5), lack pre-fast control periods. A year after Levanzin's 31 day fast he returned for 6 days of tests. The information to be gained from the report is not as to the effect of fasting as compared with the non-fasting condition but rather as to the progressive changes which occur in the course of a prolonged fast. Langfeld however concludes "The tests depending most on the muscular reactions, i.e., the strength test, showed a falling off. The tapping test which also involved the muscles but in which the rapidity of reaction was a more important factor showed no improvement. As soon as one turns however to the sensory discriminations one notices an increased efficiency which is probably due either to a change in the peripheral organs or central processes or both." In 1916 Marsh (6) reported a seven-day fast of two subjects with one week preceding and one week following during which food was respectively decreased and increased. There was no further control or practice period. His results are given in terms of their rank orders making it impossible to deduce anything as to the magnitude of his obtained differences from such derived scores, except that in the middle range of 40 to 60 they are probably very small. However it is interesting to note that he also found a decrease in strength of grip, an increase in sensory acuity, and an increase in manual steadiness. The only study in which pre-fast and post-fast control periods were recorded is that of J. A. Glaze (7). He had three subjects who fasted from 10 to 33 days. The four tests which involved what he calls mental output showed a general decrease. Manual steadiness was improved but in equilibrium or standing steadiness he was unable to demonstrate any consistent change. There was an increase in visual acuity and sensitivity to odors, Glaze (8) also noted an increase of fatiguability during fasting and a rise in both mental and muscular efficiency in the post-fast period.

METHOD. The technique of obtaining the knee jerk and its recording in the first two experiments, carried out in the fall of 1922 and in the fall of 1923, was the same as was described in connection with the insomnia study (1). In brief the stimulating device was a spring driven hammer so placed as to strike the patella ligament perpendicularly at a point one

third of its length away from the attachment to the patella. The excursion of the hammer was limited to 2 cm., and as it was placed at 1 cm. distance from the ligament the depression of the latter was the same in all cases. The kick was recorded graphically on a slowly moving kymograph by means of a writing point attached to a heel stirrup by a non-elastic cord passing over necessary pulleys with friction minimized as much as possible. The height of the excursion measured in centimeters was thus proportional to the extent of movement of the heel. The subject was seated comfortably in a Morris chair leaning back at an angle of 135°. with the leg to be stimulated hanging free from the knee and the other leg supported by an extension of the chair. There was a 10 minute rest in the chair with eyes closed before the beginning of the experiment. The blows were then given without warning at an irregular series of intervals, none being less than 15 or more than 30 seconds; an average of the first ten successive kicks was taken as the daily record. The strength and extent of the blow and its location were the same in each case, and the environmental conditions of temperature, humidity and absence of noise as well as spoken directions, were kept constant.

In the third experiment certain modifications were introduced. Instead of a reclining chair a flat table top was used, covered with a 4 inch cotton pad. The subjects lay on this with the leg supported only as far as the knee joint. From the edge of the table and opposite to the popliteal space a flexible non-elastic tape was suspended to which a wide band was attached for fastening around the leg. To the back of this band and exactly 7 inches below the table level, a non-elastic cord was attached which passed under the table and up to a metal band freely sliding on a pair of bars as in a myograph, and carrying with it in one direction only a continuous measuring tape. When the leg was extended this band was pulled along and with it was pulled the tape measure. Subsequent swinging of the leg would not be recorded as it is always less in extent than the initial kick. The experimenter setting the tape at zero or taking a preliminary zero reading could then read off directly in numerical form the extent of the excursion, push the band back to its starting point (without changing the position of the tape), and similarly record successive kicks—or merely record the sum of 10 kicks if that is taken to be the unit. The object of these modifications was to so standardize and simplify conditions that comparable records could be obtained under circumstances where Morris chairs are not available or facilities for smoking records, and also where subjects differ in size and leg length. The objection that for stout people the position on the table is not an entirely comfortable one is minimized by the use of two pillows at need, one under the waist, the other under the head or shoulders. The slight tension put upon the ligament in this position is probably approximately a constant and is an advantage since it results in the

flexion (100° to 110°) usually recognized as optimal for obtaining the knee jerk.

A further change was the substitution of an electrically driven hammer of the same type as the Johnson hammer (9). Instead of the electric release, however, as I wished to give irregularly timed blows and avoid as far as possible anticipation or a tendency to rhythmic response of psychic origin, a special switch had to be devised in which the interval between make and break of the current was controlled mechanically by a very heavy coiled spring such as is used on doors to prevent slamming. The strength of the blow was measured at frequent intervals as follows: the hammer was placed vertically above and in contact with the end of a balance, and resistance was introduced into the circuit until the hammer just failed to move a weight of 430 grams. It would be possible therefore to reproduce this stimulating strength in any other type of hammer.

In regard to the steadiness test in experiment 3 the method is the same as described in our former article (1). A graphic record on smoked paper was obtained by the moving of a metal stylus attached vertically to the subject's head over a plate suspended horizontally above him. Three successive one minute records were so taken with the subject standing in military position (heels together and toes slightly separated) with eyes closed. After some comparative study of the many possible numerical treatments of these graphic records [two of which are reproduced in (1)], the most significant was found empirically to be the sum of the least and greatest diameters. The average of three such sums in cubic meters was taken as the score.

The salivary reflex was measured by placing a dentist's ordinary glass salivary ejector in the subject's mouth connected with a graduated collecting tube. The suction was obtained by connection with running water and was kept constant by having the water flow through a given tube from a constant level. The ejector was placed under the subject's tongu and a five minute total collection was made, then a circle of 24 porcelain beads $\frac{3}{4}$ cm. in diameter on a flexible wire was placed in the subject's mouth in such a way that the beads lay inside the teeth and across the posterior part of the tongue. This generally produced a mild tendency to gagging or nausea which I have found to be the greatest stimulus to reflex salivation. With the beads in the mouth a five minute collection was made, and again a final five minute collection without the beads.

The fourteen subjects, four women and ten men, were, except two professors who served as subjects in experiment 1, medical school students and research workers in physiology. It is fair to assume that their desire to coöperate and their interest in the problem made more dependable their effort to keep the amount of sleep and general activity a constant during the experiments. The error from such uncontrolled outside sources is probably

negligible except in the case of fluid intake during fasting. As Kleitman has shown in the dog, there is a strong tendency to drink less when not eating (10). In humans the lack of thirst is aided by decreased occasion to drink when fasting and the subjects themselves reported repeated failures to maintain a standard in this regard.

The hour of the day was, with a few noted exceptions, the same for each individual subject throughout the experiments.

DISCUSSION. In eleven of the fourteen subjects fasting caused a marked decrease in the knee jerk. This was true regardless of the normal magnitude of the kick in the control periods. In subject W, experiment 3,

TABLE 1

Expt. 1. The extent of the knee jerk in centimeters (av. of 10 determinations) on successive days

	K*♂	Ha*♀	He♀	B♂	L♂	C♂
Pre-fast period		6.9				
	4.6	6.0		3.3		7.3
	3.1	8.3		4.4		5.2
	3.3	6.9		7.3		7.7
	5.6	6.9		5.5	2.0	4.4
Fasting periods	6.0	4.9			2.1	7.7
	4.1	6.6		4.9	1.0	5.6
	3.3	4.3	5.6	4.2	0.5	5.8
	2.9	3.6	2.6	2.7	0.0	6.9
	0.9	0.8	0.6	1.6		4.7
Post-fast period	4.1	3.5	4.1	5.1	0.8	
	1.2		10.0	6.1		
	5.2		8.0			
	3.5					
	3.7					

* Subject K had 32 more continuous daily records in the post-fast period and Ha in the pre-fast, but in these the range of variation is no greater than in the days here given.

a progressive decrease occurred up until the last day. On this day he was in the midst of a hunger period during the test and it was impossible to postpone the latter as was customary under these conditions. The contractions of the empty stomach have been shown by Johnson and Carlson to augment the knee jerk (11) and this is obviously the cause of the increase on this day. It is interesting to note that even so the average level on non-fasting days was not reached. The absence of a drop in knee jerk in subject C, experiment 1 and experiment 2, is less easily accounted for, but the few control records may have been insufficient here to establish the normal level. In fact we might not be justified in stating that the decrease

in fasting is greater than daily fluctuations if we did not have absolute proof in subjects K and Ha that this is the case. It is probable therefore that the same tendency shown in the shorter series in the other subjects is not due to chance.

We cannot say with equal assurance that there is a true improvement in steadiness as a result of fasting but the change though small is remarkably consistent. At any rate it is obvious that there is no loss in the ability steadily to maintain an erect position during fasting.

TABLE 2

Expt. 2. The extent of the knee jerk in centimeters (av. of 10 tests)

Figures supplied by Dr. A. H. Steinhaus

Subject 1♀		
Fasting	18 hours	7.25
	44 hours	4.54
	71 hours	5.19
Post-fast	60 hours	3.38
	1 week	5.78
Subject 2♂		
Fasting	44 hours	2.4
	69 hours	0.2
Post-fast	6 hours	0.0
	4 days	5.92
	9 days	3.82
Subject 3♀		
Pre-fast		12.7
Fasting	23 hours	2.2
	47 hours	0.3
Post-fasting	6 hours	0.0
	54 hours	8.9

We have considered standing steadiness as a true reflex in which the stimulus is the group of sensations from the leg muscles, both because of its direct dependence upon the functional integrity of the posterior columns of the cord and because it is so little subject to conscious control.

The apparatus being noiseless the subject is wholly unaware whether or not he has made a good or a bad record and in fact will more often feel sure he has done worse than usual when he has done particularly well. Apparently this is a reflex which depends rather upon the sensitivity to the stimu-

lus than upon the state of the response mechanism. One stands still if a slight deviation is corrected promptly with only the slight muscular movement necessary, rather than if it is corrected vigorously when more marked swaying has occurred. On the other hand in the knee jerk, as we measure it, we have a reflex which is primarily dependent upon its muscular component. No matter how efficient the stimulus or ready the central pathways, if the power of the muscles to lift the leg is decreased the record

TABLE 3

Expt. 3. The extent of the knee jerk in centimeters (average of 10 determinations) on successive days

	H♂	M♂**	J♂*	I♂**	W♂*,**
Pre-fast period					20.3
		5.6			19.1
		3.3		2.7	21.7
		1.9	3.8	3.6	15.1
Fasting period	0.9	2.5	1.7	2.7	19.1
	1.9	0.3	2.1	0.7	17.9
	0.8	0.0	1.1	0.0	15.7
	0.2		1.5		14.5
	0.0				18.0†
Post-fast period	0.4	2.9	5.4	1.5	22.1
	3.1	1.3	4.8	3.1	
	1.9			7.4	
	1.1				

* A series of 10 days' records of this subject taken two months later gave an average of 7.52.

** These subjects discontinued their fast after three days.

† Hunger period.

is low. If therefore, as has been reported repeatedly both objectively and subjectively, there is a decrease during fasting in muscular efficiency, whether it be neuro-muscular or purely muscular and whatever its nature, we would expect what we find here: a greater decrease in the amplitude of the knee jerk than in the degree of deflection of the head in standing steadiness.[2]

Our results in the salivary reflex show a decrease in the total 15 minute secretion in all cases. Here again two types of interpretation suggest

[2] In experimental insomnia (1) the reverse effect on both knee jerk and steadiness occurs. According to our reasoning here this would be explained by a raising of the threshold, or a change in ease of conduction of nervous impulses centrally so that a stimulus to be effective in producing a reflex must be increased in strength, with no loss of the muscular power to make the response.

themselves. The decrease may be due to changes in the nervous elements or to changes in the secreting gland. If the latter is the case and the change is entirely due to decrease in fluid intake or rate of secretion in the gland, we would expect fasting to affect all parts of the test equally, unless the gland failed entirely to secrete. If on the other hand the change is due to decreased response to the stimulating device such as would result from a raised threshold the decrease in salivation in fasting would occur especially in the second period of our test when the beads are in the mouth. If on the other hand the threshold were lowered we would expect the decrease to occur mainly in the first and third periods. This is in fact what we find

TABLE 4

Expt. 3. Daily records in the Romberg test, measured in centimeters (av. of 3 determinations)

	G	H	M	J	I	W
Pre-fast period		9.5				9.3
	6.4	8.4				8.3
	7.4	7.4	7.5			8.8
	7.5	7.4	11.0			10.4
	7.6	4.8	6.8			8.6
Fasting period	6.6	8.4	7.5			
	6.9	7.7	7.5			
	5.9	7.2	8.1		6.6	
	4.6	5.3		10.0		7.2
	7.1	6.1				6.2
Post-fast period	6.7	6.8	8.1	10.2	9.6	7.8
	7.0	9.4	8.0	11.1	11.1	
	7.0				7.9	
Av. Normal	7.09	7.67	8.28	10.65	7.20	8.87
Av. Fasting	6.22	6.94	7.70	10.0	6.6	6.70

to be the case in four out of six cases (table 6A). In only one of the other two cases is the change marked in the other direction. In other words, these two subjects are the only ones for whom the beads are not a more efficient stimulus during fasting than normally (table 6B). Obviously there is both a general and differential effect of fasting on the 3 periods, and the gross loss is the result of a local change in the gland and a change in the functional activity of the reflex arc. The latter in most cases was in the direction of an increased irritability or lowered threshold to the stimulus. This is consonant therefore with our findings of a tendency to increased steadiness in the Romberg test and lends assurance to our tentative explanation of the changes found.

SUMMARY

1. Records of the amplitude of the knee jerk were obtained on 14 subjects during three to five day fasts with preceding and following control periods which in two subjects extended over two months. In all cases there was a tendency for the knee jerk to decrease which was clearly marked in eleven of the cases.

TABLE 5

Expt. 3. Total number of centimeters of saliva secreted in 15 minute period (av. of 3–5 days)

	G	H	M	J	I	W
Pre-fast period	5.375	8.71	6.04	7.525	5.75	16.92
Fasting period	3.24	7.54	3.83	7.425	5.5	6.85
Post-fast period	3.84	13.1	5.05	10.35	4.65	9.0

TABLE 6

SUBJECT	A		B	
	Ratio between av. secretion fasting and non-fasting		Ratio between av. secretion with beads in mouth and without	
	Without beads	With beads	Non-fasting	Fasting
G	0.475	0.746	3.312	5.195
H	1.107	0.098	2.003	1.098
M	0.696	0.582	2.614	2.189
J	0.689	0.915	1.330	1.765
I	0.770	1.833	0.789	1.878
W	0.266	0.509	2.051	3.929

2. The graphic Romberg test obtained on 6 subjects during similar fast periods revealed a slight tendency to an increase in steadiness.

3. Salivation in the same six subjects decreased during fasting. In four of the subjects the decrease occurred mainly when no mechanical stimulating device was in the mouth. The response to this was relatively increased during fasting.

4. The following explanation for the above findings is suggested. The knee jerk is largely dependent upon its muscular component in our measurements of it and is here lost through change in the muscles. Steadiness, though a skeletal reflex of somewhat similar type, is dependent mainly upon the height of the reflex threshold to protopathic sensations which during fasting tends to be lowered. The gross decrease in salivation is due to changes in the gland but the differential effect is to be explained by neurological change in either the receptive or conducting mechanism.

This change is more often in the direction of an increased irritability during fasting.

BIBLIOGRAPHY

(1) LEE AND KLEITMAN: This Journal, 1923, lxvii, 141.
(2) KLEITMAN AND CRISLER: This Journal, 1927, lxxix, 571.
(3) KLEITMAN: This Journal, 1927, lxxxii, 686.
(4) LANGFELD: Psychol. Monographs, 1914, xvi, 71.
(5) LUCIANI: Das Hungern. transl. by Fraenkel, 1890.
(6) MARSH: Psychol. Rev., 1916, xxiii, 437.
(7) GLAZE: Amer. Journ. Psychol., 1928, xl, 236.
(8) GLAZE: Amer. Journ. Psychol., 1928, xl, 569.
(9) JOHNSON: This Journal, 1927, lxxxii, 75.
(10) KLEITMAN: This Journal, 1927, 336–340.
(11) JOHNSON AND CARLSON: Wis. Journal, 1928, lxxxiv, 189.

THE CONDUCTIVITY OF ERYTHROCYTES COMPARED WITH THAT OF SERUM

G. N. STEWART

From the H. K. Cushing Laboratory of Experimental Medicine, Western Reserve University

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In 1896 I discovered, while making experiments on the output of the heart by a method involving measurements of the conductance of blood, that the specific conductivity of defibrinated blood and of sediments of blood was much less than that of the serum (15 or more times less in the case of sediments) and concluded that the cells (erythrocytes) were extremely poor conductors in comparison with the liquid in which they are normally suspended. This conclusion was at once extended to embrace leucocytes (pus cells), and the cells of various solid organs from which more or less satisfactory suspensions could be obtained (scrapings of intestinal mucosa, liver, spleen, thymus, etc.). The generalization was ventured upon that this was a property common to all cells. In my first two papers (Stewart, 1897a, b) the causes and some of the implications of this property of cells were discussed. It was pointed out (Stewart, 1897b) that since some serum is necessarily present in the sediments the conductivity of the erythrocytes must be much less than the actually measured conductivity of the sediments, so that, "in comparison with serum the conclusion seems warranted that erythrocytes are non-conductors" (Stewart, 1897a). Two alternative (not mutually exclusive) explanations were suggested: 1. That the corpuscles, while capable of electrolytic conduction in their interior, are surrounded by a (relatively) non-conducting envelope or external layer, impermeable, or little permeable, to certain of the ions of blood, but not necessarily structurally differentiated from the rest of the corpuscle. 2. That throughout the substance of the corpuscle the electrolytes are combined with proteins and hemoglobin, or at least that they are hindered from dissociating normally by the presence of these substances and their ions from migrating as they would in an aqueous solution. Some evidence was brought forward that compounds of the nature suggested could be liberated from erythrocytes by certain hemolytic processes. For instance, when blood is laked by repeated freezing and thawing, the conductivity is not usually increased but may be somewhat lessened; but if distilled water is now added to the already

laked blood the conductivity is increased just as if the water had been originally added to unlaked blood. Emphasis was laid on the bearing of the observations on some of the well-known electrical phenomena exhibited by such tissues as muscle and nerve (the difference of apparent conductance in the longitudinal and in the transverse direction, electrotonic currents, and other so-called polarization phenomena, the high degree of polarizability of these tissues compared with the polarizability of the surface of contact of ordinary solutions of electrolytes, etc.).

Observations were also mentioned (Stewart, 1897b) on the conductivity of gastric juice, milk and curds, pus, cyst liquids, etc. It was pointed out (Stewart, 1897b) that a relation exists between the ratio of the conductivities of blood and serum and the proportion of serum and erythrocytes in the specimen. A simple formula was given which is approximately correct for blood rich in serum or diluted with a known porportion of serum. When the proportion of erythrocytes increases another factor than the fraction of the total volume occupied by erythrocytes influences the conductivity, namely, the fact that conduction must take place along an "out and in" path, formed by the thin threads of serum twisting among the corpuscles. The conductivity therefore diminishes more rapidly than in proportion to the increase in the fraction of the total space occupied by the corpuscles as the content of erythrocytes is increased. The curve (fig. 1) ceases to be approximately linear and becomes convex towards the abscissa axis. The relative volume of serum and erythrocytes estimated by the conductivity method was compared with that estimated by a colorimetric method (hemoglobin). In a later paper (Stewart, 1899b) rather extensive data were derived from accurately measured dilutions of erythrocytes suspended in their own serum. The proportions of serum varied from nearly 90 per cent to about 10 per cent. From these data it was shown that excellent determinations of the relative volume could be obtained under conditions which left erythrocytes and serum entirely unaltered. I have regretfully observed that most writers seem to think that the chief interest of the fundamental fact that cells have a low conductivity lies in the application of it to the measurement of the concentration of the erythrocytes. This application is interesting and useful, but the physico-chemical structure of cells on which their low conductivity depends has far wider relations and a much greater biological interest than this. I have, at various times, discussed certain phases of this question (Stewart, 1899a, 1901, 1909, etc.), and have published numerous experiments in which erythrocytes and other cells, acted upon by substances which cause hemolysis, leucolysis, etc., particularly those whose mode of action is more or less understood, have been examined as to the changes produced in their electrical conductivity, osmotic pressure and other properties. The effect of partial and complete fixation by various reagents

upon the conductivity has also been investigated in numerous instances (Stewart, 1902a, b). Results of considerable interest have been obtained by the study of the action of hemolytic, leucolytic and other substances upon the conductivity, etc., of partially and fully fixed cells. Microscopic study and enumeration of the "shadows" and measurement of their volume and the conductivity of suspensions of them in many forms of hemolysis, and with large and small doses of the active substances, have been carried out (Schroeder and Stewart, 1925). Incidentally, the so-called reversal of hemolysis (Brinkman et al., 1923–4) has been shown to be based on misinterpretation of certain appearances, as was indeed demonstrated by me (Stewart, 1902a, b) partly in association with Peskind (1902) many years ago. It is unnecessary to mention other directions in which the significance of the small conductivity of cells was explored by me, sometimes in collaboration with pupils. They are all to be found in easily accessible journals.

CONDUCTIVITY OF BLOOD SEDIMENTS. In the present paper I shall give data on the values of the ratio $\frac{k_1}{k}$ in sediments of blood centrifuged for shorter or longer periods, as a rule at a constant speed and in the same centrifuge. The specific conductivity of the sediment is represented by k and that of the serum by k_1. In some of the experiments one tube was removed after a certain number of hours and the conductivity of sediment and serum measured. Another tube of the same blood was taken out after a further period in the centrifuge. All the blood specimens were obtained from rabbits, defibrinated and immediately placed in the centrifuge. Rabbits were chosen chiefly because of the relatively large proportion of serum and the apparent ease of sedimentation. In every case the serum was completely free from hemoglobin. The ratio $\frac{k_1}{k}$ was determined for the defibrinated blood as well as for the sediment. The method of obtaining the sediment as free as possible from supernatant serum was as follows. All the serum which could be removed safely without admixture with any corpuscles was pipetted off. Then pipetting was carefully continued of the chief part of the remaining visible serum with small amounts of erythrocytes from the top of the sediment. Rolled up Swedish filter paper was then put down into the centrifuge tube till it touched the top of the sediment, left there for a couple of minutes, and then withdrawn. This was repeated about three times. The wall of the tube was kept clear of erythrocytes as far as possible by swabbing gently with filter paper. During this time the tube was in the bath, supported in a small copper gauze vessel, so that the sediment was practically at bath temperature (about 25°C.) when the conductivity tube was filled. This is important since it is certainly difficult, and possibly harmful to some of the erythro-

cytes, after the long centrifugation, to stir the extremely viscous sediment. With only one to two per cent of serum in the sediment, there may be 20,000,000 erythrocytes in the cubic millimeter. There is no need for stirring, as settling of erythrocytes in such a sediment is a process more theoretical than real. In any case the measurement is taken within a minute of the filling of the conductivity vessel, and this can be done safely if the material is already at bath temperature, as mentioned. The same is true of defibrinated blood or any dilution of erythrocytes: if the stock is already at bath temperature no stirring of the blood is necessary unless a series of observations extending over several or many minutes is required. Then, of course, the blood must be stirred. In ordinary defibrinated blood settling begins, of course, at once; in the first minute the erythrocytes settle as fast as, perhaps faster than in any other minute. But it can rarely, if ever, happen that this will introduce a sensible error if the blood is already at bath temperature and the measurement is made immediately after the tube is filled. Occasionally I have filled the conductivity vessel by pouring from the centrifuge tube the sediment, freed as much as possible from supernatant serum as described. Generally, however, another method has been preferred. A straight pipette with a wide bore has the point cut off. The pipette is closed by the finger to prevent the sediment, as much as possible, from entering it as it is passing through the upper layer of the sediment. The pipette is then introduced to about the bottom of the centrifuge tube and sediment sucked up. The blood is quickly rubbed off the outside of the pipette with a piece of filter paper and the conductivity vessel filled.

Some data are assembled in table 1 and plotted in figure 1. In the graph the first 22 points (down to the values of p, 11.5; $\frac{k_1}{k}$, 16.47 inclusive) were determined by actual measurement of the number of cubic centimeters of serum present in 100 cc. of the blood specimens. The numbers plotted as ordinates represent the cubic centimeters of serum. As abscissae are plotted the ratios of specific conductivity of serum to that of blood $\frac{k_1}{k}$. A very much larger number of points could have been inserted from the available data, without altering the curve at all. But it was desired not to overcrowd the graph so that it could be used for determining by means of a ruler the quantity p when k_1 and k were measured. The remaining part of the graph represents the continuation of the curve to the right on the same scale for values of $\frac{k_1}{k}$ up to 127.3 inclusive. This is the highest value of the ratio actually measured, the sediment having a conductivity over 127 times less than that of the serum. As some serum is necessarily included in the sediment, the conductivity of the erythrocytes

is certainly less than this. The values of p are calculated by means of formulae which fit the upper portion of the curve very closely, but the actual values of p have not been directly determined for the experiments included in table 1 and plotted in the six strips in the lower part of figure 1.

TABLE 1

NUMBER OF ANIMAL	SEDIMENT						SERUM	DEFIBRINATED BLOOD						
			Cubic centimeters of serum in 100 cc.							Cubic centimeters of serum in 100 cc. blood				Hematocrit
	$K(25°) \times 10^4$	$\frac{k_1}{k}$	(a)	(b)	(c)	F	$K(25°) \times 10^4$	$K(25°) \times 10^4$	$\frac{k_1}{k}$	(a)	(b)	(c)	F	
1	3.0	42	4.2	4.2	4.1	5.0	125.6	71.6	1.75	73.4	73.7	72.0	71.8	60 (45 m.), 63 (75 m.), 66.5 (125 m.), 67 (215 m.), 68.5 (365 m.)
2	1.6	80	2.1	2.2	2.0	2.4	126.7	62.7	2.03	67.1	66.4	64.9	65.0	60.5 (105 m.), 61 (150 m.), 62 (240 m.), 62.5 (330 m.)
3	1.15	111	1.6	1.6	1.6	1.7	128.7	63.7	2.02	67.6	66.8	65.2	65.2	49 (35 m.), 64.5 (95) m.), 65 (135 m.)
4	5.9	22.4	7.6	7.6	8.0	9.2	131.3	64.5	2.03	65.7	66.4	64.9	65.0	
5	1.5	88.6	1.9	2.0	1.9	2.1	132.0	64.8	2.04	65.9	66.3	64.8	64.8	60.5 (60 m.), 62.5 (150 m.), 63 (300 m.)
	1.0	127.3	1.3	1.4	1.3									
6	1.3	103.6	1.7	1.7	1.7	1.8	131.3	58.0	2.26	61.5	61.4	61.2	60.3	
7	1.1	123	1.4	1.4	1.4	1.4	133.4	65.4	2.04	65.7	66.3	64.8	64.8	59.5 (70 m.), 61.5 (135 m.), 62 + (200 m.), 62.0 (255 m.).
8	3.2	39.7	4.3	4.3	4.3	4.7	131.3	74.6	1.76	72.6	73.1	71.7	71.6	
9	3.3	38.2	4.5	4.5	4.5	4.9	126.1	71.6	1.76	73.4	73.1	71.7	71.6	
10	4.2	30.0	5.7	5.7	5.7	6.2	126.0							

Formula (a): $p = \frac{k}{k_1}(174 - k(5°))$. Formula (b): $\frac{k_1}{k} + \frac{1}{2} = \frac{174}{p} - \frac{3}{100 - p}$. Formula (c): $\frac{k_1}{k} + \frac{1}{2} = \frac{175}{p} - \frac{p}{400}$. The number of cubic centimeters of serum in 100 cc. of blood or sediment is represented by p. In column F are the cubic centimeters of serum calculated by Fricke's formula. Formula (c) can also be written $\frac{k_1}{k} + \frac{1}{2} = \frac{7 - p^2}{4p}$, where p is the proportion of 1 cc. of the blood which consists of serum.

If, however, these strips are imagined to be successively joined on to each other towards the right at the level of the abscissa axis of the upper part of the curve, it is seen that the curve is continued quite smoothly down to the value $\frac{k_1}{k} = 127.3$; the curve asymptotically approaching the

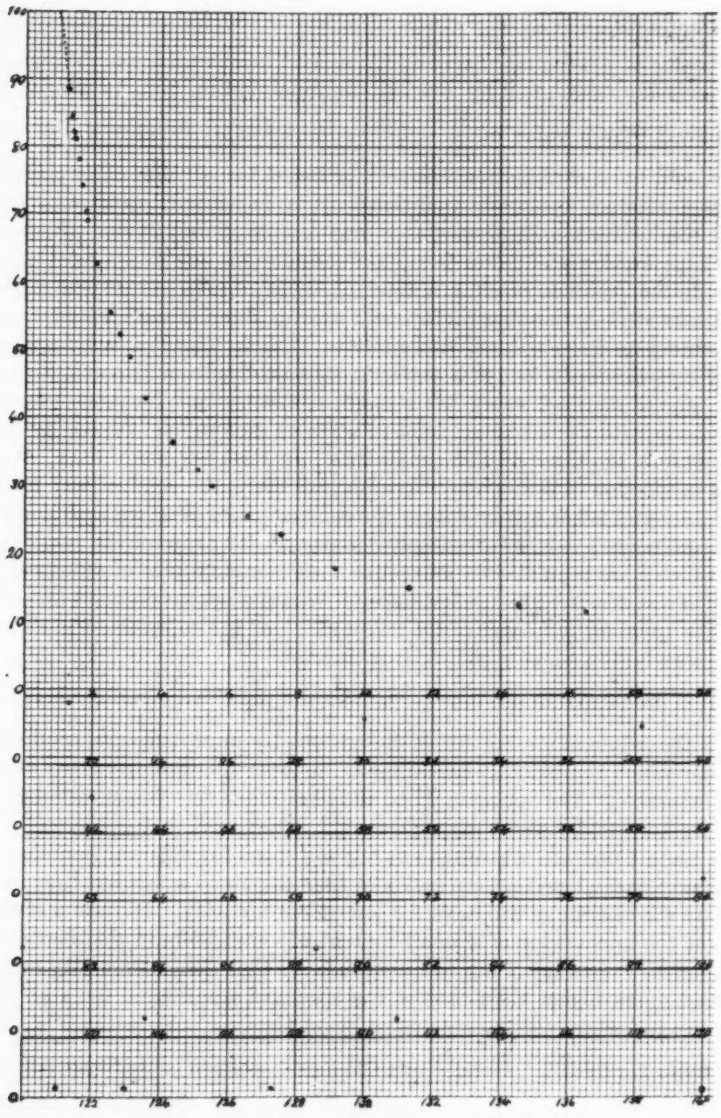

Fig. 1. Graph showing relation between the ratio $\frac{k_1}{k}$ (conductivity of serum divided by conductivity of blood) and p, the number of cubic centimeters of serum in 100 cc. of blood.

abscissa axis. One point, corresponding to $\frac{k_1}{k} = 140$, has been extrapolated, the value of p being calculated at 1.1, and 1.2 by my formulae (b) and (c) respectively, and at 1 by Fricke's formula (1924a, b). It cannot be assumed that a value of $\frac{k_1}{k} = 140$ or more will be found in a sediment obtained by centrifugation from defibrinated blood. But it is unlikely that I attained optimum conditions. It is, however, already clear that the conductivity of erythrocytes is very low compared to that of serum; perhaps hundreds of times less if we consider the relatively great effect which the presence of even a trace of serum would have. One writer (Brooks 1924–5) has thought it worth while to state that he assumes that erythrocytes have a small conductivity. The statement is not based on any measurements, but it is perfectly safe although not important, since the purest distilled water has a small conductivity. I cannot imagine anybody with the slightest claim to write on this subject supposing that these cells are absolute non-conductors. In the case of my data, where the proportion of serum was determined directly, the conductivity of the erythrocytes is not involved since the conductivities of serum and blood in the unknown specimen are simply compared with those of the standards in which p has been directly determined. The same is true of polarization, which was discussed in my first paper. Whatever polarization is involved in the apparent resistance of the unknown blood is also involved in that of the standard in which p is directly estimated.

It is probable that the maximum value obtained for $\frac{k_1}{k}$ can be increased and the minimum value for p diminished by improving the conditions under which the blood is centrifuged. A lower room temperature would be favorable. The optimum speed and duration of centrifugalization could be worked out by check experiments. I have found that if specimens of the same blood are centrifuged at a given speed for different periods it is not necessarily true that the sediment from the blood which has been longest in the centrifuge will have the lowest conductivity. Beyond a certain point the appearance of a small content of hemoglobin in the serum and diminished viscosity of the sediment indicate that the conductivity will be found much higher than that of the specimen centrifuged for a shorter period and whose serum contains no blood pigment. An instance is given in table 1, rabbit 7. Here $\frac{k_1}{k}$ was 123 for a specimen centrifuged 7 hours, and only 9.04 for a specimen centrifuged 13 hours. The second specimen was slightly laked, the supernatant serum being tinged with blood pigment. The temperature of the tubes was such that heat laking could not have occurred. The viscosity of the sediment was much less than in the first

specimen. The great increase in the conductivity of sediments by the addition of a trace of saponin, which causes complete laking, was illustrated in a previous paper (Schroeder and Stewart, 1925). The conductivity in one case rose from $K\ (25°) \times 10^4 = 3.0$ to 62.8. In blood from rabbit 3, table 1, the conductivity of the sediment increased from $K\ (25°) \times 10^4 = 1.1$ to 16.7 on addition of a trace of saponin.

It is easily seen from inspection of the curve, or of the numerical data from which it is constructed, and of other data from which other almost identical curves could be plotted (see especially, Stewart, 1899b) that there is no reason to expect a discontinuity at either end. The curve is perfectly smooth. Interpolation can be made with certainty at any point within the extreme observed values. It is evident, but has been indicated by a broken line, that extrapolation is possible at the top of the curve beyond the extreme observed value of p (about 90). In fact the broken part has been put in by laying a ruler so that the edge includes 5 or 6 of the first points, where the curve is sensibly linear, and carrying on the line till it cuts the ordinate represented by unity, corresponding to the ratio $\frac{k_1}{k} = 1$ (i.e., $p = 100$). It should be noted that this was done on a graph which has been reduced to two-fifths in the figure. Whether the extrapolated part of the curve runs exactly into the ordinate 1 at the level of $p = 100$ or not is of no consequence; it certainly comes very near to this.

Yet one writer (Brooks 1924–5) concludes after a so-called "critical examination," (*lucus a non lucendo*?) without any experimental observations whatever, that my work was "unsound" because, according to him, substitution of the extreme values $k_1 = k$ (corresponding to 100 per cent of serum) and $k = o$ (corresponding to 100 per cent of erythrocytes), in my formulae led to impossible results. In fact, as anyone with even a moderate acquaintance with elementary algebra ought to have seen, when $k = o$ all the formulae used by me give the result $p = o$. This is also true of Fricke's formula, where substitution of $k = o$ gives $\rho = 1$, i.e., 100 per cent of erythrocytes. Of course the substitution is made after a slight transformation of the equations. For instance by inverting the two sides of the equation k can be put into the numerator instead of the denominator. The same is true if $\rho = 1$ is substituted in Fricke's formula. This is one of the ordinary devices when dealing with functions which contain such a quantity as $a - x$ in the denominator of a fraction, if the value a is to be given to x. It is common to find a section on indeterminate functions in textbooks of algebra, explaining how to deal with such expressions by artifices like the one mentioned, by differentiation and in other ways. In solving the quadratic equation left after substitution of a numerical value for $\frac{k_1}{k}$ in formula (b) the negative square root is chosen. It is humiliating to have to mention these things in a scientific paper.

There is nothing disreputable about empirical formulae. They have often proved extremely useful. Mine are a little more than empirical for it was clearly seen in constructing them that they must be built around the ratio $\frac{k_1}{k}$ or its reciprocal. The

results deduced from them agree excellently with the experimental data and also with those yielded by Fricke's (1924a, b) theoretically derived formula. I agree with Brooks when he praises Fricke's work. It is perhaps at this date scarcely a breach of confidence to say that I was asked by the editor of the Physical Review, of course without Doctor Fricke's knowledge, to go over Fricke's paper for that journal (Fricke, 1924b) mainly, I suppose, because he had used my experimental data for testing his formula. The only thing I pointed out as requiring consideration was the fact that the ratio $\frac{a}{b}$ (diameters of the erythrocytes), which is involved in the determination of the constant β, was difficult to measure with any exactness and that, as a fact, our knowledge of the precise shape and magnitudes of these cells in different mammals left much to be desired. I noted, however, that Fricke's graphs showed that a considerable variation in $\frac{a}{b}$ could occur without causing a very large change in β. Brooks has given a good analysis of the uncertainties involved in measuring the diameters of erythrocytes. If I am not wrong, Fricke at present verifies his constant β to some extent by comparison of the results calculated from the formula with the experimental results. This is generally considered to be legitimate.

To return to my formulae, they were adapted, in the first instance, to fit the range of values which might be encountered in blood in health and disease. They really do more than this, but that was considered the essential thing. That a formula should give a correct value where the blood is all serum is of no importance at all for the practical purpose of determining the relative volume of corpuscles and serum in a specimen of blood, and has nothing to do with the "soundness" of the formula. We do not need to tell from a formula that we are dealing with pure serum. In fact formula (a) gives very good results up to 93 or 94 per cent. It is usually a few per cent too low when $\frac{k_1}{k} = 1$, i.e., for pure serum but not always. Formula (b) gives a close agreement up to about 90 per cent of serum. It is not difficult to construct a formula which shall give a correct result ($p = 100$) when $k_1 = k$, and also a correct result ($p = o$) when $k = o$. Formula (c) is such a formula. But in the important range there is no reason to think it any better than the other and that is why results calculated from (c) have been placed in table 1 for comparison. If there is any mathematical fault to find with (b) it is that the equation is of the third degree, whereas the other two are equations of the second degree. But as regards the practical use of this formula that makes no difference. I shall repeat here what has already been said that we can get along very well without any formula, by using the numerical data or the curves which bring us close to the underlying relations. Many instances could be given of empirical formulae fitting only a portion of a curve. For example, Regnault needed three formulae to fit different ranges of temperature for interpolating values of vapor pressure of water. Much later these three formulae were reduced to one.

Brooks' statement that my formulae "are unsound, since they give impossible values for conductance of cel's alone or suspending fluid alone" may now be dismissed as based on nothing more than his inadequate knowledge of the subject.

COMPARISON OF ELECTRICAL METHOD WITH HEMATOCRIT. As in all my previous work, I find that the relative volume of erythrocytes given by the electrical method is somewhat less than that yielded by centrifugation. This is true whatever formula is used, whether Fricke's or mine, as they all give

approximately the same values. It is scarcely necessary to say that if the values are interpolated in the tables of experimentally determined results or by measurements on the graphs, the same discrepancy is found. It is indifferent whether the hematocrit is used or the ordinary centrifuge tubes, both give the same result. Generally the discrepancy is 2 to 3 cc. in the sediment from 100 cc. of blood, the value of the sediment being usually 5 or 6 per cent too great and that of the serum about 2 per cent too small. Occasionally, the discrepancy is greater, and this is more likely to occur in specimens rich in corpuscles, and in which for one reason or another the corpuscles settle with unusual slowness. Rarely the hematocrit gives practically the same result as the electrical method, as in experiment 3, table 1, for the defibrinated blood. The reason for the discrepancy is that the electrical method gives the correct values. It cannot fail to do so if the standard estimations have been made correctly, since the erythrocytes are unaffected by the measurements and are suspended normally in the serum. The hematocrit is by no means an instrument of precision, but it is quite good enough for most clinical observations if used properly; and although the electrical measurements are simplicity itself, it is far better that persons who do not know how to make them accurately, or what to do with them when made should use the centrifuge. In table 1 under "hematocrit" are given successive values for serum as centrifugation proceeded; in means minutes from beginning of centrifugation.

The hematocrit does not yield entirely accurate values for the sediment for more than one reason. A factor which certainly contributes to the discrepancy is the serum, which cannot be got rid of completely from the sediment. Nobody, I think, will have the hardihood to assert that the sediment in the centrifuge tubes consists of dried corpuscles, or even of corpuscles whose surfaces are dry. Dried erythrocytes lake even when placed in their own serum. This was shown long ago by Stewart (1902b) and by Guthrie (1903). If the (rabbit's) erythrocytes are assumed for simplicity to be discs 6μ in diameter and 2μ thick, about 18,000,000 would be contained in 1 cu. mm. of well centrifuged sediment. A layer 0.01μ thick around an erythrocyte would have a volume of about $0.95\mu^3$. For 18,000,000 erythrocytes the total volume of the layer would be about 17,000,000 μ^3 or 0.017 cu. mm. of serum in 1 cu. mm. of sediment, i.e., 1.7 per cent of serum. This is about the proportion calculated as present in well centrifuged sediments in table 1. A thickness of 0.01μ would be one-three hundredth of the radius of the disc-shaped corpuscle or about 30 times the diameter of a molecule of carbon dioxide (Nernst). The layer of serum in contact with each erythrocyte on the assumption made is accordingly very thin. If it were 5 times as thick, i.e., 0.05μ, the volume of the layer would be 0.082 cu. mm. in 1 cu. mm. of sediment, or 8.2 per

cent of serum. This is approximately the proportion of serum calculated for the least well centrifuged sediment in table 1.

Access of such quantities of oxygen as the cells in a sediment could use up if conditions for oxygen use were normal, and exit of such quantities of carbon dioxide as they produce must be rendered difficult or impossible in a tightly packed sediment in the bottom of a centrifuge tube. The sediment is seen to be dark after prolonged centrifugation. This factor and others which may influence the blood gases in the sediment may cause changes in the volume of the erythrocytes, although it is impossible to say what share, if any, they take in producing the discrepancy between the hematocrit and the electrical method.

There is of course deformation when the corpuscles are packed down by long centrifugation. Nobody knows whether this is associated with an increase in average volume due to the taking up of liquid. A change of this kind might be a factor in the observed, small discrepancy. In any case, if deformation occurs the relation between surface and volume will be altered, unless the change is balanced by migration of water. Some hemolysis can be caused by prolonged centrifugation when precautions have been taken against such rise of temperature as would cause heat laking. This has been referred to already in connection with the conductivity of sediments.

In obtaining sediments for making the dilutions in my standards for conductivity determinations only moderate centrifugation was employed, 10 per cent or more of serum being left in the sediments. It was not necessary to centrifuge exhaustively because it was desired to produce only such erythrocyte concentrations as could exist in blood. A check on the sediment dilutions was also afforded by the numerous observations on uncentrifuged defibrinated blood, in which of course there was no possibility of any change in the erythrocytes. The range of concentration of erythrocytes in the specimens of blood examined is nearly as wide as for the dilutions of sediments. Curves of the same kind can be plotted from the results on blood. They coincide closely with those plotted from the data on sediment dilutions and fit the formulae as closely. It can therefore be assumed with confidence that my standards were not sensibly affected by changes in the volume of the sediments due to deformation of the erythrocytes by centrifugation.

"Critical examination" of my experimental data. Brooks tries to show that my results understate the volume of erythrocytes contained in the various dilutions. He does not bring forward any experimental data to check up with mine. He simply sits at a desk and writes "If." "If," he says, in my estimations by a colorimetric method of the residual serum in the sediments with which I start in making the various dilutions by the addition of measured quantities of serum from the same blood, some of the

hemoglobin employed passes into the erythrocytes, the serum volume in the sediment will be overestimated and the erythrocyte volume underestimated. He gives no evidence whatever that normal erythrocytes take up hemoglobin, but cites Brinkman's (1923–4), observations on pseudoreversal of hemolysis as showing that shadows after laking can do so. This is called a critical examination. Brinkman's conclusion is due, as already mentioned, to misinterpretation of certain microscopic appearances and is without foundation. But supposing it was true, how could it be assumed from this that intact erythrocytes loaded with the normal stock of hemoglobin would absorb hemoglobin from a solution of it in serum when placed in contact with it for a minute or two?

I used another method, that of Hoppe-Seyler, to determine the serum in the sediments in order to have a double check. In a number of the experiments both methods were applied to the same sediment. The agreement in the results was very satisfactory. This was considered to entitle the data to greater confidence. According to Brooks, this was a complete delusion. For "*if*" in the washing of the sediment, entailed in Hoppe-Seyler's process, hemoglobin (and proteins) came out of the corpuscles the volume of corpuscles would be underestimated. It had to be underestimated so as to account for the discrepancy with the hematocrit results without admitting that the hematocrit usually gives sediment volumes which are rather too high. *If* I had employed a third method it might have been somewhat puzzling to know which direction the hemoglobin ought to take, but I have confidence that Brooks would have found a way. As in the case of the colorimetric determination, he made no observations to prove whether a sufficient loss of material from the erythrocytes to influence the results sensibly could actually occur. He italicizes the word "wash" in speaking of Hoppe-Seyler's method, as if that was a reproach. Yet washing erythrocytes is a well-understood and extremely common process. All the serologists in the world use it. I have washed erythrocytes hundreds of times without seeing anything remotely resembling the disturbing picture conjured up in the "critical examination." I admit that erythrocytes can be badly washed, but persons who do not know the proper technique ought not to be washing erythrocytes; they could be harmlessly employed as critics.

Brooks' analysis of one of my experiments in which the serum was determined in duplicate by a colorimetric method and also in duplicate by Hoppe-Seyler's method illustrates the thoroughness of his criticism. He does not mention the important point that the duplicate colorimetric determinations gave 58.843 cc. serum and 59.711 cc. respectively in 100 cc. blood (Δ = 1.47 per cent) while the duplicate Hoppe-Seyler determinations gave 59.051 cc. serum and 58.480 cc. respectively (Δ = 1.03 per cent). Mean of the duplicates by colorimetric method 59.277; by Hoppe-Seyler's

method 58.765 (Δ = 1.15 per cent). It should be remembered that in the colorimetric determination over 93 per cent of the serum by volume was directly measured by centrifugation; it was only less than 7 per cent of residual serum which was estimated colorimetrically. There was obviously no chance for any serious error here. The Hoppe-Seyler estimation, which agrees within about 1 per cent with the colorimetric estimation is also clearly an accurate determination; otherwise there could be no such agreement. The mean of the Hoppe-Seyler and colorimetric estimations is 59.02 cc. of serum; the electrical method gave 59.39 by one formula and 59.73 by another (mean 59.56, or only 0.9 per cent more than the mean of the colorimetric and Hoppe-Seyler determinations). Is it not clear that such an agreement between the results of these three methods could only be attained if all three were right? Is it not ridiculous for a critic, who professes to analyze this very experiment, to sit down at his desk and prove that if a certain amount of the protein and hemoglobin of the cells was lost in washing, the amount of serum should have been more than 10 cc. less in 100 cc. of blood than I found it? What the experiment really proves is that if any loss of protein and hemoglobin occurred it was so insignificant that it produced no sensible effect on the serum estimation. A ten per cent error would have been revealed by a gross disagreement with the other methods. It is surely childish to insist that if an error was committed, which the experiment shows could not have been committed, the serum would have been wrongly estimated by over 10 per cent, whereas the data prove that it was correctly estimated.

But let it be admitted for a moment that the erythrocytes in the sediment were underestimated by both methods, how does it happen that the degree of underestimation is the same, so that an excellent agreement in the results of the two methods is obtained? How can the quantity of hemoglobin taken up by the erythrocytes in the colorimetric estimation be such as to give the same underestimation of the erythrocyte volume as the quantity lost in washing in Hoppe-Seyler's method? There is no connection between the two; it is quite impossible that in a number of experiments these quantities should accidentally agree. The true explanation of the agreement of the results of the two methods of estimation is that they both measure the proportion of serum and erythrocytes with satisfactory accuracy.

Again, it is impossible to see, if the discrepancy between the results of the hematocrit and the electrical method are due to underestimation of the erythrocyte volume in the sediments, how the data obtained by the electrical method should usually come so near those obtained by the hematocrit and should never exceed the hematocrit values, being nearly always a little less, as they ought to be. It is absurd to suggest that this could be due to any approximately constant, small underestimation by two totally

different methods, hemoglobin migrating into the erythrocytes in the one case, and out of them in the other in the proper proportions to produce just the difference of 2 or 3 cc. or less of erythrocytes in the 100 cc. of blood between the hematocrit and the electrical determinations.

It might have been supposed that the close agreement of the values calculated from Fricke's formula with my experimental data would have been a point in favor of the accuracy of the latter. But Brooks does not think so, and he concludes that the agreement does not help the experimental data at all. They remain just as "unsound" as they were, perhaps more so. The formula, on the other hand, which agrees with the experimental data, emerges without a stain on its character, after certain cosmetic operations by Brooks. My own opinion is that neither Fricke's formula nor my data can either gain or lose by this kind of critical examination, which however, does get pretty tedious.

CONDUCTIVITY OF INTERIOR OF ERYTHROCYTES. Many years ago Höber attempted to estimate the conductivity of the interior of erythrocytes. Fricke's more recent work has advanced our knowledge of that difficult matter. He finds that the conductivity is about one-third that of serum. If it is assumed that hemoglobin is present in the erythrocytes to the amount of about 30 grams in 100 cc. of the moist corpuscles, and that it is in such a condition as to depress the conductivity of the erythrocyte contents in the same proportion as it would that of serum (Stewart, 1899a), this factor alone would account for a reduction of the conductivity of the intracorpuscular liquid from a value equal to that of serum to a value less than half that of serum. It is therefore quite possible that the intracorpuscular liquid if taken separately from the hemoglobin might have a specific conductivity not much inferior to that of serum.

Let K (25°) $\times$ 10^4 = 150 for serum after eliminating the depression due to proteins. Assume this value for the protein-free and hemoglobin-free intracorpuscular liquid. Deducting the diminution of conductivity caused by 8 per cent of proteins and, say, 32 per cent of hemoglobin in 100 cc. of intracorpuscular liquid, we get about 65 as the value of K (25°) $\times$ 10^4 for the interior of the corpuscles, i.e., between one-half and one-third the value for serum. A result which seems to follow clearly from Fricke's estimate is that the low specific conductivity of erythrocytes is not due mainly to a low conductivity of the interior. The alternative is a poorly conducting outer layer or envelope, and there is good evidence that this is present in many cells, including erythrocytes.

SUMMARY

The specific conductivity of well centrifuged sediments of blood has been found to be considerably less than one-hundredth of the conductivity of the serum ($\frac{1}{106}$, $\frac{1}{111}$, $\frac{1}{123}$, $\frac{1}{127}$ in 4 specimens of blood). In two specimens

it was $\frac{1}{80}$ and $\frac{1}{88.6}$ respectively; and values ranging from $\frac{1}{30}$ to $\frac{1}{42}$ were found in 4 specimens. There is reason to believe that still lower values than the lowest measured can be obtained for sediments; and it is certain that the larger conductivites could have been lowered by better choice of the speed and duration of centrifugation. It must be remembered that even in the best separated sediment some serum is present, so that the actual conductivity of the erythrocytes is no doubt much less than that measured in any of the sediments. The presence of 1.3 per cent of serum in the sediment showing the lowest conductivity would account for its conductivity. This proportion of serum would be more than furnished by a layer of serum around each erythrocyte 0.01μ in thickness, which would correspond to about 1.7 per cent.

Inspection of the data or of the graphs plotted from them, an example of which is given in figure 1, shows that if extrapolated beyond the point corresponding to 90 per cent of serum the curve will cut the ordinate representing a ratio of $\frac{k_1}{k} = 1$, corresponding to 100 per cent of serum, approximately at the 100 per cent line. This must be so if the experimental data from which the graphs are plotted are correct, as there is no doubt they are. At the other end the curve approaches the abscissa axis asymptotically as $\frac{k_1}{k}$ increases. The maximum value which can be actually measured for $\frac{k_1}{k}$ has not been determined. The estimation of the conductivity of erythrocytes alone is complicated by the existence of some serum in the sediment, after the most thorough centrifugation. If $\frac{k_1}{k}$ reaches a maximum which cannot be exceeded, the corresponding point on the curve will be slightly above the abscissa axis.

Beyond a certain point continued centrifugation may be associated with a great increase in $\frac{k_1}{k}$ compared with that found in the same blood at an earlier stage. When some hemolysis occurs in prolonged centrifugation, the serum, previously free from hemolgobin, becoming tinged, the ratio of the conductivity of the serum to that of the sediment increases. This is due to an increase in the conductivity of the sediment, with practically no change in that of the supernatant serum. The viscosity of the sediment diminishes at the same time.

The addition of a trace of saponin in substances to a sediment greatly increases the conductivity (to 20 times or more) and diminishes the viscosity.

My previous conclusion that the hematocrit yields somewhat too high values for the sediment is confirmed. Reasons are given for this.

BIBLIOGRAPHY

BRINKMAN, R. AND A. V. SZENT-GYORGYI. 1923–4. Journ. Physiol., lviii, 204.
BROOKS, S. C. 1924–5. Journ. Gen. Physiol., vii, 349.
FRICKE, H. 1924a. Journ. Gen. Physical., vi, 741.
1924b. Physical. Rev., xxiv, 575.
GUTHRIE, C. C. 1903. This Journal, viii, 441.
NERNST, W. 1913. Theoretische Chemie, p. 464.
PESKIND, S. 1902. This Journal, viii, 99, 404.
SCHROEDER, M. AND G. N. STEWART. 1925. This Journal, lxxiii, 505.
STEWART, G. N. 1897a. Journ. Boston Soc. Med. Sci., 1897, June 15, no. 16, p. 18.
1897b. Centralb. f. Physiol., 1897, xi, 332.
1899a. Journ. Physiol., xxiv, 211.
1899b. Journ. Physiol., xxiv, 356.
1901. Journ. Physiol., xxvi, 470.
1902a. Journ. Med. Res., viii, 268.
1902b. This Journal, viii, 103.
1909. Journ. Pharm. Exper. Therap., i, 49.

PHYSIOLOGICAL VARIATIONS IN THE CARDIAC OUTPUT OF MAN

VI. The Value of the Cardiac Output of the Normal Individual in the Basal, Resting Condition

ARTHUR GROLLMAN

From the Laboratory of Physiology, School of Medicine, The Johns Hopkins University

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Despite the obvious physiological and clinical importance of knowing the value of the cardiac output of the normal human individual, the difficulties and errors involved in the methods used for determining the cardiac output have heretofore precluded the possibility of obtaining this value. The present paper reports the results of a series of determinations of the cardiac output, pulse, oxygen consumption and, in certain cases, of the blood pressure of fifty-one individuals selected at random from a group of young, normal individuals. This series comprises the greatest number of cases heretofore reported and is sufficiently large and of a degree of accuracy to permit the evaluation of the normal cardiac output of man. The data also are evidence for the constancy of the cardiac output of man and the possibility of predicting a value for the normal individual. This ability to predict the cardiac output is particularly important for the clinical applications of determinations of the cardiac output, for it permits one to compare a given determination with the average normal value and thus conclude as to the degree of its abnormality.

Methods. For the determination of the cardiac outputs, acetylene was used as described in previous publications (Grollman, 1929a,b,c,d). The most likely cause of error in the use of this method is a failure on the part of the subject to mix properly in the rebreathing procedure. The criteria for complete mixing have been described in a previous paper (Grollman and Marshall, 1928) and the ease with which failure to attain complete mixture may lead to errors has been demonstrated (Grollman, 1928). However, to a certain group of individuals, acetylene has a very unpleasant taste despite the description of a "not unpleasant, ethereal odor" which chemical treatises (Meyer and Jacobson, 1907) attribute to the purified gas. Such subjects may fail to breathe deeply and thus render even more difficult the attainment of correct results. It is, therefore, essential to insure that the breathing has not been influenced by the subject's reactions and that complete mixture has been attained. It is quite prob-

able that in certain subjects, particularly certain clinical cases, homogeneous mixture is incapable of attainment under the conditions of the experiments and hence correct values will not be obtainable.

The subjects for the present determinations were all apparently normal and healthy individuals between the ages of about 20 and 30 except subject 46 of table 1 who was 42 years of age. They were, in practically all cases, medical students, and being acquainted with the general procedure used, were not subject to any psychic factors which might possibly affect the normalcy of the results obtained (Grollman, 1929d). The determinations were made in the early morning immediately on the arrival of the subjects at the laboratory. They were performed in the post-absorptive state and hence may be considered as basal. The determinations were made in the sitting posture after the subject had rested comfortably in a steamer chair for at least one-half hour. The subjects, in practically all cases, lived within a few minutes' walk of the laboratory and hence were not subject to any great exertion before the determination. In order to avoid the effect of variation of temperature on the cardiac output (Grollman, unpublished results) the room in which the determinations were made was maintained at 22° ±1°.

The pulse rate was determined by palpation for 30 seconds: the blood pressure, by the use of the Tycos recording sphygmomanometer; and the oxygen consumption by the use of the Krogh metabolism apparatus.

Results. The results of this investigation are given in the accompanying table. In column 5 of the table are given the surface areas of the subjects as read from Dubois (1924) charts.

The average pulse rate observed in the present study (column 6) was 64 with a variation from 49 to 78. The pulse rate under normal conditions thus appears to be the most variable of the several circulatory functions and bears little, if any, relationship, probably, to the cardiac output.

The blood pressures recorded in column 7 of the table are much lower than those usually accepted as the average of normal persons. This is to be attributed to the complete state of relaxation and rest in which the subjects were studied.

In column 8 is given the oxygen consumptions observed which are compared to the calculated values as given in column 9. The latter are calculated, according to the Aub-DuBois Standards, on the basis of 39.0 Calories per square meter of body surface for men and 37.5 Calories, for women. The oxygen equivalent of the Calorie under basal conditions has been taken as 4.813. The average deviation of the observed from the calculated oxygen consumptions is 3 per cent. This probably is also to be attributed to the complete state of relaxation attained on the part of the subjects. The most marked deviation of the observed from the calculated values was shown by subject 38 whose basal metabolic rate (as

TABLE 1.

The pulse rate, blood pressure, oxygen consumption, arterio-venous oxygen difference and cardiac output of normal men and women

SUBJECT NUMBER	SEX	HEIGHT	WEIGHT	SURFACE AREA	PULSE	BLOOD PRESSURE	OXYGEN CONSUMPTION		ARTERIO-VENOUS OXYGEN DIFFERENCE	CARDIAC OUTPUT	CARDIAC OUTPUT PER UNIT AREA OF BODY SURFACE
							Observed	Calculated			
		cm.	*kgm.*	*sq. meters*		*mm. mercury*	*cc. per minute*	*cc. per liter*	*cc. per liter*	*liters per minute*	*liters per sq. meter*
1	♂	164	68	1.73	70	106/65	235	237	58	4.05	2.34
2	♂	170	77	1.87	68		260	256	60	4.33	2.32
3	♂	180	72	1.90	64		236	260	61	3.87	2.04
4	♂	168	57	1.63	66	102/65	226	223	60	3.77	2.31
5	♂	178	74	1.91	62		243	261	65	3.74	1.96
6	♀	140	63	1.49	64		198	191	59	3.36	2.25
7	♀	171	62	1.71	60		244	219	60	4.07	2.38
8	♀	171	54	1.62	78		219	208	57	3.84	2.37
9	♀	177	63	1.77	60		248	227	61	4.07	2.30
10	♂	177	60	1.73	64		220	237	62	3.55	2.05
11	♀	155	49	1.45	64		158	186	59	2.68	1.85
12	♂	182	78	1.98	68		256	272	56	4.57	2.30
13	♂	179	78	1.95	51		265	267	62	4.27	2.29
14	♂	180	58	1.73	58		195	239	59	3.30	1.91
15	♂	181	71	1.90	50		256	260	64	4.00	2.11
16	♂	171	68	1.78	66		229	246	57	4.02	2.26
17	♂	183	71	1.91	66		244	262	62	3.94	2.06
18	♂	180	67	1.84	62		223	251	57	3.91	2.13
19	♀	158	55	1.54	60	98/63	207	197	67	3.09	2.01
20	♂	178	59	1.72	60	103/63	218	240	55	3.96	2.30
21	♂	173	72	1.84	63	107/67	246	254	64	3.84	2.09
22	♂	178	68	1.84	60	108/65	220	254	63	3.49	1.90
23	♂	181	93	2.13	75	116/74	300	295	65	4.61	2.16
24	♂	168	57	1.63	70	98/62	206	223	60	3.43	2.10
25	♂	185	81	2.04	60	112/72	260	280	61	4.26	2.09
26	♂	167	61	1.68	60	107/67	226	230	60	3.77	2.24
27	♂	173	67	1.78	60	103/69	230	246	58	3.97	2.23
28	♂	187	68	1.91	70	103/65	245	264	58	4.22	2.21
29	♂	178	62	1.76	62	115/80	230	243	56	4.11	2.34
30	♂	163	56	1.59	55	106/72	205	218	55	3.73	2.35
31	♀	165	65	1.70	60	100/65	226	218	62	3.65	2.15
32	♂	184	75	2.02	76	98/60	280	276	63	4.44	2.20
33	♂	178	62	1.76	68	101/64	230	241	56	4.11	2.34
34	♂	181	70	1.88	62	110/73	264	257	65	4.06	2.16
35	♂	169	61	1.69	60	113/73	202	231	59	3.42	2.02
36	♀	175	71	1.85	74	103/73	247	240	59	4.19	2.26
37	♀	177	65	1.79	66	107/67	231	229	56	4.13	2.31

TABLE 1—*Concluded*

SUBJECT NUMBER	SEX	HEIGHT	WEIGHT	SURFACE AREA	PULSE	BLOOD PRESSURE	OXYGEN CONSUMPTION		ARTERIO-VENOUS OXYGEN DIFFERENCE	CARDIAC OUTPUT	CARDIAC OUTPUT PER UNIT AREA OF BODY SURFACE
							Observed	Calculated			
		cm.	*kgm.*	*sq. meters*		*mm. mercury*	*cc. per minute*	*cc. per liter.*	*cc. per liter*	*liters per minute*	*liters per sq. meter*
38	♂	174	60	1.71	69	102/63	280	234	59	4.74	2.77
39	♂	171	63	1.72	50	109/70	213	235	60	3.55	2.06
40	♂	181	67	1.85	60	108/68	245	253	56	4.38	2.21
41	♂	177	56	1.70	66	115/67	252	233	60	4.20	2.47
42	♂	183	67	1.86	58	103/64	254	254	60	4.23	2.27
43	♀	150	57	1.51	70	100/62	198	193	55	3.60	2.38
44	♀	156	48	1.44	78	98/63	163	184	55	2.96	2.06
45	♀	164	53	1.56	72	103/63	180	200	58	3.10	1.99
46	♂	175	75	1.90	51	100/60	254	260	65	3.91	2.06
47	♀	156	56	1.54	74	108/65	199	197	58	3.43	2.23
48	♂	162	64	1.67	60	123/78	228	228	55	4.15	2.49
49	♂	178	74	1.91	58	110/70	245	261	54	4.54	2.38
50	♀	165	52	1.55	74	90/58	209	199	55	3.80	2.45
51	♂	160	59	1.60	49	102/67	190	219	56	3.39	2.12
Average				1.76	64		230	237	59.3	3.88	2.21
Average deviation from mean									2.6		0.14
Average percentage deviation from mean									4.4%		6.4%

found on repeated determinations) was +20 per cent. This subject was probably abnormal and suffering from a mild degree of hyperthyroidism. Subject 11, also, was very probably abnormal and should not be included in the present series. Her basal metabolic rate was −15 per cent and she showed other signs of hypothyroidism. With the exception of these two subjects, all others were apparently normal and healthy.

The most outstanding and remarkable finding of the present investigation is the relative constancy of the arterio-venous oxygen difference as shown in column 10. The average deviation of this function from the mean was only 4.4 per cent. The conclusions to be drawn from this finding will be discussed in a later section.

In column 11 are given the cardiac outputs which are seen to vary from 2.68 liters in the case of subject 11 to 4.61 in the case of subject 23. The cardiac output being the quotient of the oxygen consumption divided by the arterio-venous oxygen difference, will be determined by these latter functions. Since the arterio-venous oxygen difference is relatively constant, and since the oxygen consumption is directly proportional to the

surface area, it follows that the cardiac output like the oxygen consumption will be a function of the surface area. This relationship is shown in the last column of the table.

Comparison of present results with those of previous investigators. Previous attempts to establish a normal value for the cardiac output of man have resulted in the attainment of two diametrically opposed series of results. Certain methods have indicated an arterio-venous oxygen difference of about 60 with consequently low values for the cardiac output. Other methods have given results which are much higher and correspond to an arterio-venous oxygen difference of about 30 or 40. Still other investigators have reported values which range from the low to the high values mentioned above. The confusion resulting from the above conflicting results have led many to the belief that the cardiac output of man is not amenable to prediction or correlation with any other data such as, for example, the body surface.

One may conclude from the present study that the cardiac output, per unit area of body surface, of normal men and women is relatively constant under fixed standard conditions and hence is predictable for such conditions. This conclusion is quite different from that of most previous investigators who have found marked diversity in the results obtained. Unfortunately, no large series of cardiac outputs have hitherto been reported, due, no doubt, to the difficulties involved in such determinations. Hence no close comparison of the present data with previous work is possible.

In a previous paper, Marshall and the author (1928) reported data on 16 individuals. These determinations were made by the use of nitrous oxide and ethylene and no precautions were taken to avoid psychic disturbances, temperature fluctuations, etc., which were later shown (Grollman, 1929b, c, d) to affect the basal values of the cardiac output. Moreover, as was shown in the original presentation of the use of acetylene for cardiac output determinations (Grollman, 1929a), this gas permits the attainment of results whose probable error is only one-fifth of that obtained by the nitrous oxide method. The combination of these factors, whose significance was not appreciated, led to the variations in the cardiac output which these authors observed.

Field, Bock, Gildea, and Lathrop (1924) also attempted to establish the rate of the circulation of the blood in normal resting individuals. From the wide range in values which they obtained, they concluded that "concerning the normal circulation rate under basal conditions . . . . the contenders for a high and a low resting blood flow are both right." Thus, if one calculates the average cardiac output per square meter of body surface for the 8 individuals studied in table 7 of their paper, one finds this function to vary from 2.47 to 6.11 or over 200 per cent. Such

erratic results seem very improbable and are due, it is believed, to the general dfficulty of obtaining correct values by the method used. This subject has been considered in a previous paper (Grollman, 1928) where the marked effects of posture on cardiac output, as found by Field and Bock (1925), were discountenanced. The results reported in the present investigation refute the contention of Field, Bock, Gildea, and Lathrop (1924) that variations in the cardiac output are to be expected and that many factors exist which are beyond control of any attempt to secure basal conditions.

Burwell and Robinson (1924) found the cardiac output per square meter of body surface to be within the range of 1.9 to 2.5 (as found in the present study) in 8 of their 11 subjects. Higher values (2.8, 3.5, and 3.7) were obtained on the other 3 subjects. Hayasaka (1927), in 7 normal subjects, found the cardiac output per square meter of body surface to vary from 1.9 to 2.6 with an average value of 2.2 which is in accord with the results of the present study. In view of the findings of the present investigation, the objection of Burwell and Smith (1929) to Hayasaka's use of the cardiac output per minute per square meter of body surface as a standard for comparison is unfounded. Nor, on the basis of the results of Burwell and Robinson (1924) quoted above, can the use of such a standard be considered as having been "shown to be an uncertain one."

The constant arterio-venous difference. The constancy of the arterio-venous oxygen difference may be interpreted as an indication of the tendency on the part of the organism to maintain a relatively constant internal environment, in so far as the oxygen supply of the tissues is concerned. However, it appears certain that the arterio-venous oxygen differences of the different tissues or organs are not the same and it is improbable that the blood supply to the same tissue or organ in different individuals is constant. Hence, one would not expect the arterio-venous oxygen difference for different individuals to be absolutely constant. The variations actually observed are, therefore, probably real and not due entirely to the errors involved in their determination.

The results of the present study establish the value of this arterio-venous difference for normal individuals as 60 ± 5 cc. per liter of blood. This corresponds to an oxygen utilization of about one-third. By assuming 60 as the probable arterio-venous difference and calculating or determining the oxygen consumption of an individual one can, therefore, predict the cardiac output for this individual within ± 10 per cent. In the case of the 2 abnormal subjects studied, in whom there was a probable derangement of the thyroid, the arterio-venous oxygen difference did not deviate from the normal. There is thus apparently an effort on the part of the organism to maintain a constant arterio-venous difference and hence this difference cannot be taken as a criterion of the normalcy of the circulatory

system. This fact was also demonstrated in the study of the cardiac output during the malaise and fever following the injection of typhoid vaccine. Despite an obvious deviation of the cardiac output from normal during such malaise, the arterio-venous difference remained relatively constant (Grollman, 1930).

The cardiac output per unit area of body surface. If one omits the results on subjects 11 and 38, whose abnormal condition has been noted above, one finds that the cardiac output of the normal individual per square meter of body surface is 2.2 $\pm$0.3. This value permits us to calculate and predict the normal cardiac output of an individual from his height and weight and gives a standard of comparison by which the normalcy of any single determination may be gauged. The importance of possessing such a standard, particularly in the case of the clinical study of the circulation, is obvious, for it permits us, for the first time, to apply the same criteria to the circulatory system as have been applied with such success in metabolic studies.

The fact that results of such a high degree of constancy have been obtained in the present study, although not a direct proof, nevertheless, adds weight to the view that with the methods employed, the true cardiac output of man is being measured. This constancy also indicates that the cardiac output for any given individual from day to day, in the resting, basal condition (with due regard to such factors as psychic disturbance, temperature, etc.) is constant. That such a constancy actually exists will be shown in future communication.

Discussion. We must conclude from the results of the present investigation that the lower values, hitherto reported by other workers, for the cardiac output of man in the basal condition, are of the correct order of magnitude. Moreover, it may be concluded that such values bear a direct relationship to the active mass of the individual as expressed by the surface-area. *Per unit of surface area, the heart output is relatively constant for normal, young, individuals when proper precautions are taken to maintain the required standard conditions.*

Although the data presented here are insufficient to permit the denial of a difference in the cardiac output of males and females, it indicates that such a difference, if it occurs, is very small.

The present study has been confined to a single age-group and no conclusions can be drawn as to the cardiac output of individuals not in their third decade of life. In fact, a few determinations have indicated that the arterio-venous oxygen difference increases with age and that the cardiac output per square meter of body surface, consequently, decreases during the life of the individual.

In conclusion, it must be emphasized that the results obtained in the present study can only be obtained when all due precautions are taken

to avoid all known factors which affect the cardiac output. Such factors are excessive quantities of fluids (Grollman, 1929b), food (Grollman, 1929c), psychic disturbances (Grollman, 1929d), and temperature variations of the surroundings (unpublished results). Moreover, assurance must be made that none of the many pitfalls which beset the determination of the cardiac output of man, vitiate the results obtained.

SUMMARY

A study was made of the cardiac output, pulse rate, blood pressure, and basal metabolism of 51 individuals in the resting, basal condition. The results indicate that the cardiac output of normal men and women is a function of their surface area and can be predicted with a high degree of accuracy. The cardiac output per square meter of body surface under fixed standard conditions was found to be 2.2 $\pm$0.3, with an average deviation from the mean of 5 per cent. The physiological implications of the results and their practical importance, was discussed.

BIBLIOGRAPHY

Burwell, C. S. and G. C. Robinson. 1924. Journ. Clin. Invest., i, 87.

Burwell, C. S. and W. C. Smith. 1929. Journ. Clin. Invest., vii, 1.

Du Bois, E. F. 1924. Basal metabolism in health and disease. Philadelphia and New York, p. 142.

Field, H., A. V. Bock, E. F. Gildea and F. L. Lathrop. 1924. Journ. Clin. Invest., i, 65.

Field, H. and A. V. Bock. 1925. Journ. Clin. Invest., ii, 67.

Grollman, A. and E. K. Marshall, Jr. 1928. This Journal, lxxxvi, 110.

Grollman, A. 1928. This Journal, lxxxvi, 285.

1929a. This Journal, lxxxviii, 432.

1929b. This Journal, lxxxix, 157.

1929c. This Journal, lxxxix, 366.

1929d. This Journal, lxxxix, 584.

1930. Journ. Clin. Invest.

Hayasaka, E. 1927. Tohoku Journ. Exper. Med., ix, 401.

Meyer, V. and P. Jacobson. 1907. Lehrbuch der Organischen Chemie, 2nd ed. Leipzig, i, 852.

THE EFFECTS OF CO_2 AND FIXED-ACID ACIDOSIS ON CONDUCTION IN THE MAMMALIAN HEART

CARL J. WIGGERS AND M. GARCIA BANUS

From the Department of Physiology, Western Reserve University Medical School, Cleveland, Ohio

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Studies on the perfused hearts of cold and warm blooded animals have amply demonstrated that the fundamental properties of the heart are affected by changes in the pH of the perfusion fluid. In general, the optimum pH apparently varies somewhat in different preparations and is affected also by many secondary factors. A reasonable decrease beyond an established optimum seems to act as a depressant while an increase tends to improve the reactions of cardiac muscle. We need only recall, among others, the investigations of Borrino and Viale (1912), Clark (1913), Mines (1913), Boehm (1914, 1922) Andrus (1919, 1925, 1926), Burridge (1920, 1921, 1927), Andrus and Carter (1922, 1924, 1926), Drury and Andrus (1924), Iwai (1924), Salant and Johnston (1924), H. Frederique (1925), Carter and Dieuaide (1926), Gremels and Starling (1926), and Smith (1926). These observations, together with information of a supplementary nature, have tended to crystallize the belief that a decrease in intracellular pH due to perverted cellular metabolism or to an interference with blood supply is ultimately responsible for depressed or aberrant functions of the heart, such as apparently develop spontaneously both in experimental animals and man. Thus Meakin and Long (1926, 1927) and also Eppinger, Kisch and Schwartz (1927) have voiced the belief that the gradual depression of contraction associated with cardiac failure and decompensation is most logically referred to such phenomena; while Andrus and Carter (1926) suggest that it may prove basic in explaining the development of "spontaneous" abnormal rhythms. Such interpretations are particularly plausible in view of the findings of Katz, Kerridge and Long (1926) that the buffering capacity of cardiac muscle is low in comparison with that of skeletal muscle.

It still remains to be established that changes in the pH of the blood due to *general acidosis* can affect cardiac functions in the whole animal in the same manner as when excess acid formation occurs intracellularly. There are several indications which make it doubtful whether a decrease in blood pH still compatible with life does affect the functions of the heart in a similar manner:

1. Depression of cardiac functions during the course of general clinical acidosis or during experimental acidosis in animals is chiefly an abrupt terminal event.

We have had frequent opportunity to observe these effects in our student and research laboratories for the past four years. When dilute mineral or organic acids are slowly introduced by intravenous injection, death of cardiac origin is always sudden; it usually occurs without much warning or gross evidence of previous depression of any cardiac function. When the exposed heart is observed its action seems to remain normal until, quite suddenly, its size increases, its contraction visibly diminishes, its rate decreases appreciably and the blood pressure falls rapidly to a low level. Frequently, though not always, partial A-V block occurs at such a crisis. Somewhat later, the contraction waves are seen to spread as ripples over the auricular surface and fail to reach the ventricles. Complete ventricular standstill then results, for the power of developing an idioventricular rhythm has apparently been lost. Artificial stimuli applied to the auricle also fail to be conducted to the ventricle, and very strong shocks must be applied to it directly in order to provoke even feeble contractions. These are visible evidences that all functions of the heart are depressed by a decrease in pH which is clearly incompatible with life. It is important, therefore, to ascertain whether changes in pH of the blood compatible with life also affect the functions of the heart within the body to a degree which is significant but perhaps not obvious by ordinary methods of study; or, whether the heart is protected in some way until an actual crisis is reached.

2. The demonstration that comparatively small changes in pH of an artificial solution produce alterations in the functions of an isolated heart is in itself not conclusive evidence that equivalent changes in the pH of the natural blood affect the intact heart similarly. There are obvious differences, a few of which may be briefly mentioned.

a. The absence of colloids, hormones, and other constituents of the blood in saline solutions commonly used for perfusion may affect the osmotic equilibrium and perhaps the permeability of cardiac muscle so that the intracellular pH is more readily altered by changes in perfusion fluids than by corresponding variations in the pH of the blood.

b. Changes in pH of whole blood are attended by alterations in bicarbonate concentration such as do not occur in artificial perfusion solutions. This in itself may modify the pH of the cell interior, for Smith (1926) has shown that bicarbonate ions diffuse readily into the cells.

c. Alterations in the pH of whole blood or of an artificial perfusate are accompanied by changes in the ionic calcium, and this may have a specific surface effect on cardiac muscle, apart from any changes in intracellular pH. While Andrus and Carter (1926) have shown that such changes in

ionic calcium cannot account for the effects produced by changing the pH of perfusates, it remains to be demonstrated that this also holds for whole blood, where equivalent changes in pH must produce much greater changes in ionic calcium, since the amount of un-ionized calcium is greater in the blood. Furthermore, in the whole animal pH changes may lead to variations in the total blood calcium.

d. The automatic denervation of the perfused heart can also not be totally disregarded, particularly as changes in the pH of whole blood may in addition affect the centers governing the action of the heart.

We have no desire to minimize the valuable information that the perfusion method is capable of yielding; on the contrary, we appreciate the advantage of controlling the number of variables and recognize with other investigators that it is probably the best experimental method for producing intracellular pH changes somewhat comparable to those occurring naturally from excess acid production within cardiac tissue. Notwithstanding, it is of equal importance to question whether a decrease in pH of the whole blood within the limits tolerated by the animal as a whole produces similar changes in intracellular pH and in consequence depression of functions.

PRINCIPLES OF METHOD EMPLOYED. The physiological effects of pH changes may be studied in a variety of ways. Changes in conduction rate or conduction time have, however, proved particularly satisfactory, not only on account of the ease and reliability with which they can be carried out but also because the conduction time is apparently not affected by secondary dynamic changes of the circulation (Wiggers, 1927a). For such studies, the P-R interval of a standard electrocardiogram has frequently been employed. This method has its limitations because the initial elevation of the P wave cannot always be determined with the necessary exactness. It is preferable, therefore, to obtain direct leads from the heart by suitable methods.

When small variations in time intervals are concerned, it is important for each investigator to satisfy himself as to the limitations of accuracy of his apparatus and method used under the conditions employed. It is generally believed that the use of comparators by which readings can be carried to a fourth decimal or beyond materially increases the accuracy of readings. We are unable to agree with this conclusion. On the contrary, the number of decimal places to which readings are ordinarily carried engenders a sense of accuracy not warranted by a check of the method. The comparator method relies on a proportionality of distances between two points on a curve and between two time lines. Obviously the shorter the distances compared, the greater the accuracy. This is so because the constancy of film speed demanded by the comparator method is not attainable over distances greater than 10 mm. in any form of

photokymograph known to us. Using an Elliott comparator on our photokymograph records we have found figures in a third decimal place undependable when conduction times greater than 0.1 sec. were concerned. We therefore found it necessary, and in fact regard it preferable for all work of this nature, that a *continuous time record* be inscribed by a tuning fork. This necessitates that the speed of the photosensitive surface be increased to 80 or 100 mm. per sec. By counting accurately the total number of vibrations included between two points and evaluating mathematically fractions of vibrations toward each end of a series, readings to within 1 to 2σ can be made. We have, however, allowed a possible error of 3 to 4σ, and can attach no real significance to smaller values either in our own data or in those of others.[1]

PROCEDURE. All experiments were performed on anesthetized dogs under artificial respiration, with the heart fully exposed but continually moistened with saline solution. Although previously reported observations by one of us (Wiggers, 1927a) had demonstrated that changes in ventricular filling or aortic resistance are without effect on conduction rate, this had not been crucially determined at the time when these experiments were carried out. Hence, the diastolic pressure was definitely controlled as described elsewhere (1928). The intraventricular pressure was also recorded optically in order to allow for possible changes in contraction and initial tension; but subsequent work showed this to be unnecessary. To obviate the effects of changing heart rates on conduction (cf. Wiggers, 1928) an artificial rhythm was established by application of regularly repeated break induction shocks to the right auricle. As in previous work (1927) the unipolar method of leading from various points on the ventricular surface was adopted. As a rule deflections from 4 to 6 points were recorded in rapid sequence. They served as multiple checks by which possible technical errors could be detected, and provided a factor of safety in case unrecognized difficulties in the registration of true intrinsic deflections crept in. Usually, the injury caused by a superficial stitch proved to be without effect on the derivation of unipolar leads; occasionally, however, the curves from one or more points were found to be erratic and had to be discarded.

Control tests made on animals for experimental periods of 3 to 4 hours showed great constancy, conduction-times varying from 3σ to 4.5σ at the most. The values obtained over different areas of the ventricle were also usually in consistent relation to each other.

Changes in pH were produced either by a slow infusion of N/4 lactic or

[1] It is perhaps fitting for the senior author to acknowledge his indebtedness to Prof. W. P. Lombard, under whose guidance he was first trained in the careful use of this method. After 25 years of trial he is convinced of its superiority over any existing method of calculation.

N/5 hydrochloric acid, or by gradually increasing the amount of CO_2 in the inspired air. In the former method the rate of infusion was so adjusted that the pH of the animal's blood was reduced from 7.3 or above to the neighborhood of 7.0 in 20 to 30 minutes. Between 60 and 140 cc. of dilute acid were required in different animals. When changes in blood pH were produced by inhalation of CO_2 the animal was sometimes over-ventilated for a brief period so as also to obtain somewhat lower values of alveolar CO_2 tension. Gradually increasing amounts of CO_2 were then added to the inspired air. At various times, a complete set of intrinsic deflections were recorded with each set of records, a reading of the alveolar

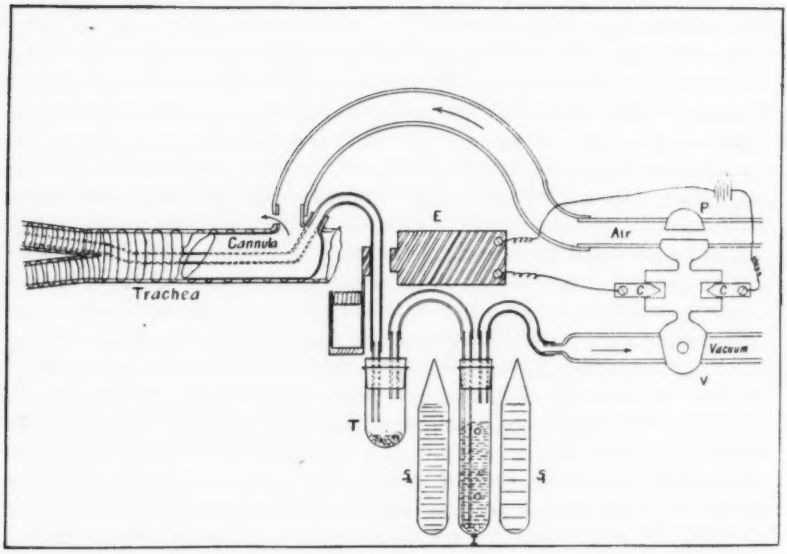

Fig. 1

CO_2 was made, a sample of blood was drawn from the carotid artery and its pH was determined electrometrically at the same temperatures and under the same CO_2 tensions as existed at the time in the alveoli. The technic actually used was recently described by one of us (Banus, 1929).

The methods for sampling the alveolar CO_2 continuously and for equilibrating the blood have already been reported in a preliminary communication (Banus, 1926). In order to understand the arrangement adapted to these experiments it should be stated that artificial respiration was maintained by regular interruption of a stream of compressed air, a buffer can being introduced to obviate undesirable mechanical effects of too

sudden a blast. Through a side communication controlled by a pressure gauge and needle valve, varying amounts of CO_2 were admitted from a tank.

In order to gauge the CO_2 content of the respired mixture continuously, samples of alveolar air were automatically drawn through a standard bicarbonate and phenol red solution at the end of each expiration only, i.e., after the artificially inflated lungs had collapsed fairly completely and had thereby expelled their alveolar contents into the bronchi and trachea. Comparison with color tubes—constructed to range from 8 to 60 mm. CO_2 tension—enabled us to express the changing CO_2 mixtures directly in terms of alveolar CO_2 tensions rather than in terms of per cent CO_2 in the inspired air,—a customary but less accurate method in mammalian and human experimentation.

A sketch of the automatic arrangement for sampling the alveolar air displaced into the bronchi and trachea during deflation is shown in figure 1. A catheter, communicating in succession with a trap, *T*, (for mucus) the bicarbonate-indicator solution, *I*, and a vacuum system, *V*, was inserted through a small side-tube of the tracheal cannula so that its end actually entered one bronchus. The valves *V* and *P* in the vacuum and air pressure lines were so arranged that the former opened for 1 or 2 seconds after the latter had been previously closed for 2 or 3 seconds, thus allowing the lungs to deflate through an opening in the tracheal cannula. Experience showed that the intratracheal pressure during deflation is sufficiently positive, however, to force a small amount of tracheal air into the comparison tube, thus decreasing the true reading of CO_2. Hence it proved necessary to shut off the tracheal catheter during the entire phase of inflation. This was accomplished by compressing the tubing in front of the trap by an electromagnet system, *E*, actuated and timed by rotating contacts, *CC*, on the valves *V* and *P*.

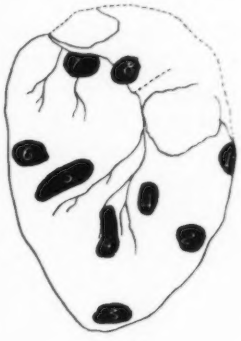

Fig. 2

Results. The records used for calculating the time intervals required for transmission of the impulses from a stimulated spot on the right auricle to various surface points on the ventricles are so similar to those previously published in other investigations (cf. Wiggers, 1927a, figs. 7 and 8) that they need not be reproduced. Figure 2 shows the position, extent and numbering of the ventricular areas within which punctate leads were placed in different experiments. Tables 1 and 2 give the results of typical experiments. In addition to the time intervals required for conduction of the impulse to various points on the ventricular surface, the tables give

data as to changing P-R intervals, calculated from an electrocardiogram,[2] (lead II) and, in some cases, conduction time to a remote point on the right auricular appendix (column A). In the last two columns the alveolar CO_2 tension and blood pH are entered. Whenever data are omitted in these tables, reliable measurements could not be made on the records.

TABLE 1

Effects of fixed acids on conduction

EXPERIMENT		P-R	A	1 –	2	3	5	7	ALVEOLAR CO_2	pH OF BLOOD
N/5 HCl										
									mm.	
C 361	IX	0.122			0.155	0.149	0.139	0.152	18	7.45
	X	0.130			0.160	0.160	0.148	0.158	35	7.01
	XI	0.130			0.170	0.160	0.152	0.162	38	Less than 7.01
C 362	VII	0.150	0.078		0.171	0.168	0.158	0.178	33	7.14
	IX	0.160	0.082		0.182	0.171	0.151	0.180	33	Less
	XI	0.160	0.086			0.176	0.170	0.182	38	7.01
C 365	I	0.154			0.181	0.178	0.161	0.169	28	7.27
	II	0.160	0.083		0.189	0.183	0.161	0.174	28	Less
	III	0.164	0.090		0.192	0.190	0.168	0.182	33	7.05
N/4 lactic acid										
C 358	XI	0.160	0.083		0.197	0.187	0.161	0.175	26	7.30
	XIII	0.163	0.083		0.197	0.190	0.168	0.176	26	Less than 7.30
	XIV	0.160	0.082		0.191	0.191	0.166	0.178	23	7.08
	XV	0.158	0.080		0.190	0.174	0.155	0.167	23	7.08
C 366	VI	0.119			0.136	0.125	0.128	0.136	23	7.33
	VII	0.118	0.074		0.138	0.129	0.131	0.138	22	7.32
	VIII	0.113	0.076		0.141	0.130	0.130	0.139	23	7.15
	IX	0.113			0.140	0.129	0.129	0.135	23	7.15
	X	0.120			0.141	0.130	0.133	0.138	18	7.11

A perusal of the first set of data incorporated in table 1 (expt. C-361, 362, 365) shows that the gradual infusion of dilute HCl caused a reduction in pH to a figure just above 7 and, in spite of our efforts to keep the CO_2 constant, a slight increase in alveolar CO_2 tension. In experiment 361,

[2] The difficulty of accurately determining the rise of P at the speed of film used, together with possible position changes resulting in the exposed heart, make it probable that too great reliance cannot be placed upon some of the P-R intervals determined from the electrocardiogram.

the final effect was obtained by infusion of 110 cc. N/5 HCl in 30 minutes, in experiment 362 by infusion of 60 cc. in 2 minutes, and in experiment 365 infusion of 40 cc. in 2 minutes. The values representing conduction-times

TABLE 2

Effects of increasing and decreasing alveolar CO_2 tensions on conduction

EXPERIMENT	P-R	A	1–	2	3	5	7	ALVEOLAR CO_2	pH OF BLOOD
								mm.	
C 357 II	0.137	0.071		0.150	0.150	0.135	0.147	26	7.29
V	0.152	0.076		0.173	0.170	0.149	0.160	57	6.96
C 363 I	0.152	0.092		0.173	0.165	0.155	0.170	26	7.29
III	0.166	0.100		0.201	0.180	0.169	0.172	60	6.80
C 353 II	0.135		0.150	0.153	0.160	0.147	0.157	22	7.27
V	0.130		0.161	0.155	0.160	0.150	0.152	41	7.06
VII	0.125		0.160	0.157	0.158	0.153	0.150	41	7.06
IX	0.133		0.165	0.160	0.165	0.153	0.155	52	6.88
XIII	0.140		0.160	0.153	0.160	0.148		23	7.30
C 352 X	0.120			0.145	0.123	0.120	0.113	57	6.93
XIII	0.110		0.137	0.132	0.119	0.116	0.125	28	7.30
C 366 II	0.130	0.084		0.156	0.145	0.142	0.150	43	7.05
IV	0.122	0.080		0.140	0.132	0.132	0.142	28	7.28
V	0.125	0.079		0.144	0.132	0.132	0.144	28	7.28
C 356 II	0.124		0.153	0.148	0.146	0.137	0.157	28	7.30
III	0.127		0.156	0.150	0.148	0.137	0.152	28	7.30
V	0.130		0.160	0.155	0.146	0.135	0.152	52	7.10
VIII			0.166	0.162	0.150	0.145	0.162	28	7.31
C 358 VII	0.163	0.087		0.199	0.190	0.163	0.181	57	6.95
IX	0.165	0.083		0.201	0.192	0.168	0.187	28	7.30
X	0.160	0.083		0.197	0.187	0.161	0.175	2[illegible]	7.30
C 360 II	0.115		0.140	0.136	0.136		0.120	42	7.0
III	0.114		0.149	0.137	0.131	0.119	0.119	42	7.0
V	0.112		0.146	0.140	0.141			18	
VI	0.108		0.138	0.137	0.134	0.120	0.120	18	7.28
C 361 I	0.122	0.063		0.155	0.151	0.142	0.160	52	7.09
IV	0.126	0.062		0.159	0.150	0.142	0.160	28	7.45
VI	0.130	0.065		0.151	0.149	0.140	0.159	28	7.45

to various surface areas of the auricle and ventricle as well as the P-R intervals increased so consistently that one can infer only that the conduction time definitely increased.

Similar effects were also obtained in many experiments in which reduction in pH was produced by increasing the alveolar CO_2 tension (table 2). An appreciable increase in CO_2 tension produced rather rapidly acts to prolong the conduction times to various surface areas. Experiments 357 and 363 are good examples of such a change. Conversely, we found in several experiments in which the animal started by breathing high CO_2 mixtures that a subsequent reduction of CO_2 quickly restored the pH toward normal and reduced the conduction intervals. The data of experiments 352 and 366 illustrate such changes. Broadly interpreted, the observations of experiment 353 may be regarded as showing evidence of both of these changes. If we focus our attention initially upon the conduction intervals existing at pH 6.88 and compare them with those found before and after (series II and XIII) we note that the values in all leads derived from surface areas are larger at pH 6.88. It is true that the P-R intervals failed to alter in a consistent manner, but this may be explained by the special difficulty in determining the rise of the P deflection with the precision desirable.

Upon a reasonable number of observations similar to these we concluded in a preliminary communication (1926) that a decrease in pH of the whole blood, whether due to fixed acids or rebreathing of CO_2 mixtures, prolongs the conduction times from the auricle to the ventricle. Since that report was made additional records have been measured, certain errors in measurement due to the use of an Elliott comparator have been corrected and the significance of all tabulated results have been reconsidered at periodic intervals. As a result of this re-evaluation of our data, we are compelled now to report other findings of a negative character, which make it necessary to amend the sweeping character of our preliminary conclusions. In the first place, prompt changes in conduction corresponding to an increase or decrease in alveolar CO_2 or blood pH were not always obtained. A tendency in this direction is obvious in the results of experiment 353. The conduction interval failed to lengthen when the pH had been reduced to 7.06 by administration of CO_2 (cf. II with V and VII); only when the pH had been reduced to 6.88 did the characteristic lengthening of the conduction interval appear. The data of experiment 356 illustrate another case in which moderate changes in pH failed to affect the conduction intervals consistently. In this instance, a slight prolongation existed in leads derived from areas 1 and 2 on the left ventricle and in the electrocardiogram, but the tendency was in the direction of a decrease in leads 3, 5 and 7. These and similar observations in other experiments emphasize 1, that sometimes very considerable changes in blood pH must be produced by CO_2 before significant reduction in the conduction time obtains, and 2, that all portions of the heart are not necessarily affected to the same degree or equally easily. Furthermore,

when the conduction intervals were increased in consequence of the administration of CO_2, they did not always return promptly to normal when the CO_2 was withdrawn; on the contrary the intervals often continued to lengthen further. This after-effect is illustrated by the tabulated data of experiments 358, 360 and 361. In the first of these experiments there was a primary tendency for the conduction interval to lengthen and in the last very little change was noted. Had observations been carried further, a subsequent reduction might have been discovered as was the case in experiment C-360.

Finally, we were never successful in demonstrating a similar lengthening of the conduction time in those experiments in which the pH was correspondingly reduced by injection of N/4 lactic acid. These effects are illustrated by the results of the last two experiments incorporated in table 1. In experiment 358, 140 cc. N/4 lactic acid were injected during a period of 19 minutes with a drop in the pH to 7.08. During the course of the injection (cf. IX with XI and XIII) the conduction intervals *decreased* rather than increased and this tendency seemed to continue throughout the period of infusion until in series XV a significant reduction was obvious.

In experiment 366, 103 cc. N/4 lactic acid were introduced over a period of 103 minutes. The conduction periods in leads derived from areas 2, 3 and 5 showed a very slight initial lengthening, but the facts that a contrary tendency occurred in leads derived from areas 5 and 7 as well as in the P-R interval, and that the difference was no greater than in the previous normal controls (VI and VII), led us to question the significance of such slight differences as occurred.

This failure to find a similar prolongation in conduction-times after injection of lactic acid suggests a natural protection of cardiac tissue from great increases in organic acids such as occur in fixed-acid acidosis, a phenomenon which may account for the absence of disturbances in cardiac rhythm and conduction in general clinical acidosis. As to the nature of the protective mechanism we can only speculate. In best accord with the known facts of general physiology, is the assumption that changes in conduction are mediated by changes in intracellular pH. Since CO_2 diffuses readily into cells (Jacobs, 1920) but the H ions as such do not (cf. Taylor, 1928), the prolongation of conduction time following use of HCl may be attributed to the passage of HCl either as an undissociated molecule or by coincident passage of the H and Cl ions. Our observations that the characteristic lengthening of conduction intervals failed to obtain when lactic acid was used to produce acidosis may then be referred to the fact that the lactate ion penetrates less easily (cf. Smith, 1926) with the result that changes in intracellular pH do not follow those of the blood.

SUMMARY

1. Changes in the pH of the blood up to a range of acidity still compatible with life were produced by slow intravenous injections of N/4 lactic and hydrochloric acids or by addition of various quantities of CO_2 to the air breathed.

2. A new method is described by which the CO_2 tension of the alveolar air was constantly read.

3. Changes in cardiac conduction were determined by measuring the conduction intervals from a point of artificial stimulation on the right auricle to various areas on the auricular and ventricular surface, the unipolar method of lead being adopted.

4. Our results were definite in showing that a decrease in blood pH to about 7.0 produced either by breathing increasing volumes of CO_2 or by slow injections of dilute HCl generally causes a prolongation of conduction times between the auricle and ventricular surface. Similar reduction of pH by infusion of lactic acid failed to show this characteristic lengthening of the conduction intervals.

5. The last observations may explain the fact that disturbances of rhythm and conduction are uncommon in clinical or experimental acidosis previous to the terminal circulatory crisis.

6. Evaluation of these results in the light of known facts of general physiology supports the belief that pH changes in the blood due to organic acids other than CO_2 do not readily produce a depression of conduction by changes in intracellular pH.

BIBLIOGRAPHY

ANDRUS, E. C. 1919. This Journal, lxviii, 221.
1925. Journ. Physiol., lix, 361.
1926. Skand. Arch. f. Physiol., xlix, 81.
ANDRUS, E. C. AND E. P. CARTER. 1922. This Journal, lix, 227.
1924. Heart, xi, 97.
1926. Journ. Clin. Invest., iii, 555.
BANUS, M. G. 1926. This Journal, lxxvi, 215.
1929. This Journal, lxxxviii, 709.
BOEHM, R. 1914. Arch. f. exper. Path. u. Pharm., lxxv, 229.
1922. Arch. f. exper. Path. u. Pharm., xcv, 1.
BORRINO, A. AND G. VIALE. 1912. Arch. di fisiol., x, 537.
BURRIDGE, W. 1920. Journ. Physiol., liii, 269.
1921. Journ. Physiol., lv, 111.
1928. Quart. Journ. Exper. Physiol., xviii, 1.
CARTER, E. P. AND F. R. DIEUAIDE. 1926. Johns Hopkins Hosp. Bull., xxxix, 99.
CLARK, A. J. 1913. Journ. Physiol., xlvii, 83.
DRURY, A. N. AND E. C. ANDRUS. 1924. Heart, xi, 389.
EPPINGER, H., F. KISCH AND H. SCHWARTZ. 1927. Das Versagen des Kreislaufes.
FREDERIQUE, H. 1925. Arch. internat. de physiol., xxv, 153.
GREMELS, H. AND E. H. STARLING. 1926. Journ. Physiol., lxi, 297.

IWAI, M. 1924. Pflüger's Arch., ccii, 356.
JACOBS, M. H. 1920. This Journal, liii, 457.
KATZ, L. N., P. T. KERRIDGE AND C. N. H. LONG. 1926. Proc. Roy. Soc., xcix, 26.
MEAKINS, J. C. AND C. N. H. LONG. 1926. Trans. Assoc. Amer. Phys., xli, 297.
1927. Journ. Clin. Invest., iv, 273.
MINES, G. R. 1913. Journ. Physiol., xlvi, 1.
SALANT, W. AND R. L. JOHNSTON. 1924. Journ. Pharm. Exper. Therap., xxiii, 373.
SMITH, H. W. 1926. This Journal, lxxvi, 411.
TAYLOR, N. W. 1928. Journ. Gen. Physiol., xi, 207.
WIGGERS, C. J. 1927. This Journal, lxxx, 1.
1927a. This Journal, lxxx, 12.
1928. Pressure pulses in the cardiovascular system. Longmans, Green, London, p. 120.

A STUDY OF THE DIRECT EFFECT OF HYPERCAPNIA ON THE CONTRACTION OF THE MAMMALIAN VENTRICLE

CARL J. WIGGERS

From the Department of Physiology, Western Reserve University Medical School, Cleveland, Ohio

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PREVIOUS WORK. In spite of extensive experimental work as to the effects of CO_2 on the dynamic changes in the mammalian heart and circulation, it can still not be asserted whether an increase in blood CO_2 above the normal exerts a stimulating or a depressing effect upon the mammalian ventricles or whether it has no specific action.

In 1908 Y. Henderson found that acapnia produced by hyperventilation caused a progressive acceleration of the heart, thereby reducing the interval of diastolic filling, and in consequence the systolic discharge. Similar effects were later noted by Dale and Evans (1922). Subsequently (1909–10) Henderson also found the rate of diastolic filling retarded owing to a diminished venous return, this in turn being attributed to depression of the "veno pressor" mechanism. Henderson throughout his series of papers alludes to the diminution in systolic and diastolic size thus occasioned as a condition of hypertonus, from which the inference may be drawn that acapnia has a direct effect on the heart itself. The writer has searched Henderson's writings in vain for such an interpretation; on the contrary the cardiac effects are assigned to secondary effects of rate and filling.

The effects of increasing amounts of CO_2 have been studied by many investigators. Jerusalem and Starling (1910) working with the heart-lung preparation observed that small quantities of CO_2 (2–8 per cent) added to the inspired air increased the systolic discharge and the diastolic size, and augmented the calculated minute output in spite of a slight coincident slowing. Similar changes in cardiac volume and systolic discharge were also found by Mathison (1910), Itami (1912) and more recently by Eppinger, Hess and Schwartz (1926). All observers appear to agree that these are effects of low degrees of hypercapnia and that excessive amounts of CO_2 act to depress the heart. But the limits at which such reversal obtains are not entirely agreed upon.

Other observers—and these appear to be in the majority—are convinced by their experiments that hypercapnia has no stimulating action. Thus Ketcham, King and Hooker (1912), working with perfused mammalian

hearts, found an increase in diastolic size but a diminished amplitude of contraction. Patterson (1914) reached similar conclusions as a result of cardiometer experiments on dogs with a natural circulation. Studies on minute volume changes by gasometric methods have also failed to show any stimulating effects. Thus the experiments of Lindhard (1915), Liljestrand (1919), Campbell, Douglas, Haldane and Hobson (1913), Schneider and Truesdell (1922) on man and the more recent observations of Marshall (1926) on unanesthetized dogs all showed that the minute volume was never increased as a result of rebreathing smaller percentages of CO_2, but was distinctly diminished when greater concentrations were inhaled. Opposed to this, stand the recent observations of Eppinger, Kisch and Schwartz (1927), who found by similar methods that the minute volume was greater and that the diastolic size studied by means of x-ray and orthodiagraphic methods was increased.

Whenever such dissimilar reactions are obtained we should make an effort to discover the cause of the discrepancies rather than to perpetuate the conflicting reports by merely adding to them. Several investigators have ventured suggestions as to why their results failed to square with those of others. A few may be recalled. Ketcham, King and Hooker (1912) pointed out that changes in venous return to the left heart were possibly not taken into account by Jerusalem and Starling. Mines (1913) attempted to harmonize the contradictory data on the basis that too alkaline a perfusate or blood was used by those who obtained stimulating effects with the result that a shift of the pH to a level nearer the optimum occurred. But this would not account for the stimulating effects obtained on intact animals. It may be suggested that some of the differences are explainable by the use or absence of anesthesia, particularly as Marshall (1925) has found that anesthesia by morphine or urethane alters the relations between heart rate and minute volume in dogs and since Eppinger, Kisch and Schwartz (1927) reported that the pressor effects of CO_2 obtained in curarized dogs are replaced by depressor effects in anesthetized animals. Aside from the fact that contrary reactions of investigators who employed the same or similar anesthetics are not explained in this way, it is exceedingly improbable that anesthesia fundamentally affects the response of the heart to chemical or mechanical agents. It is conceivable, as Eppinger, Kisch and Schwartz point out, that very deep anesthesia may depress or abolish the normal excitability of the vasomotor centers with the result that peripheral dilatation from local actions of CO_2 replaces a vasoconstriction of central origin. But such a fall of blood pressure is not ordinarily observed as an effect of CO_2 in anesthetized dogs. On the contrary, vasoconstriction and rise of blood pressure appear to be the customary effects [cf. Bayliss (1906), Kaya and Starling (1909), Mathison (1910), Itami (1912)]. Recently Gollwitzer-Meier (1929), who also investigated the

effects of CO_2 on intact animals concludes that certain fallacies creep in during inhalation of CO_2 mixtures because reflexes inaugurated from the lungs affect the circulation independent of changes produced by alteration of blood CO_2

As far as the writer is able to evaluate the experimental conditions of previous workers, it appears that aside from the investigations of Ketcham, King and Hooker (1912) on perfused mammalian hearts, the question whether CO_2 has a *direct* effect on the mammalian ventricle has not been critically tested. As the writer (1927) recently pointed out, the cardiac effects of chemicals and drugs may be mediated through a primary or direct influence on the heart or they may be *secondary*, i.e., be produced by alterations in venous return, arterial resistance or heart rate. Since increasing arterial CO_2 produces alterations in all of these factors, most of the experimental effects, including those on unanesthetized animals and on man, represent a resultant of such secondary changes in which the direct effect may be completely obscured. This applies also to investigations made by means of the heart-lung preparation, in spite of the belief that venous return and arterial resistance are controlled. The writer (1928, p. 120) and Katz (1927) have repeatedly pointed out that the use of this preparation is apt to inspire a sense of security not warranted by the physical arrangement of the system. Ketcham, King and Hooker have alluded to its failure to control left auricular filling. The records of Jerusalem and Starling give graphic evidence that considerable changes in arterial pressure occurred in spite of the supposedly automatic control of arterial resistance. The right auricular pressure likewise is not kept constant, especially when coronary flow alters. The fact that blood in a venous reservoir is maintained at a constant level is no guarantee that mean right auricular pressure remains the same and constancy in the latter is not evidence that diastolic filling pressures are unaltered. Finally, changes in heart rate are apt to occur when the CO_2 concentration is varied.

Procedure. It was the purpose of this investigation to reëxamine the extent to which direct and secondary effects contribute to the cardiodynamic alterations produced when CO_2 of the blood was increased to the upper tolerable limits. The observations were made on dogs' hearts *in situ* and in *controlled circulation* experiments, i.e., while the heart rate was automatically controlled, and tendencies of arterial resistance and venous return to change were compensated. The method by which arterial pressures and heart rate were controlled have been described previously (1927). To this was added a simple expedient for controlling the venous pressure. Whenever venous pressure increased as a result of CO_2 it was compensated by slightly constricting the vena cava inferior; when it tended to fall traction was reduced.

Changes in alveolar and blood CO_2 were created, controlled and measured

in the manner recently described in association with Banus (1929). As a rule, the alveolar CO_2 tensions were gradually increased to 55 or 60 mm. Determinations of pH were omitted in these experiments, but as conditions of experimentation were comparable to those of the preceding investigation we are safe in assuming that the pH decreased approximately to 7.0 or 6.9, i.e., to a level at which it was shown in the preceding paper distinct depression of conductivity occurred. To avoid the possibility that the addition of CO_2 to the inspired air might reduce the O_2 content of the blood, a generous admixture of O_2 from a tank was made so that the oxygen of the inspired air equalled 30 to 50 volumes per cent during all the tests.

The pressures in the left ventricle and aorta were optically recorded according to methods described in detail elsewhere (1928). Venous pressures were read in the left auricle by an adaptation of the principle suggested by Henderson and Barringer (1913). Care was taken that this pressure should not fall below 45 mm.; usually it was above this.

Results. This report is based on 19 experiments in which intraventricular pressure curves were studied as to effects on initial tension, amplitude and duration of systolic phases. In selected instances, the surface areas of subdivisions of the curves were also compared according to the procedure of the author and Katz (1928). The aortic pressure curves were studied as to the constancy of diastolic pressure and as to possible variations in pulse pressures or contour.

Since in experiments of this sort conclusions can be based only on the analysis of records, far too numerous for detailed presentation, it is desirable to preface the presentation of illustrative curves by a statement of the general conclusions reached:

Increasing or decreasing the alveolar CO_2 betwen the ranges of *15* to *60* mm. tension usually has no demonstrable *direct* effect on the dynamics of the heart beat in the dog. In two experiments ever so slight a tendency either to depress or to stimulate the heart was recognizable, but these effects were insignificant in comparison with changes that CO_2 produces secondarily through alterations in peripheral resistance, venous return or heart rate. In the following description those conclusion are illustrated by specimen records and specific discussions.

Figure 1 (C-455-III) contains four segments of aortic and left intraventricular pressure curves taken when alveolar CO_2 readings were 12+, 45, 55 and 55 mm. respectively. In the first three segments, right auricular pressure registered 46, 45 and 45 mm. saline respectively. No alteration in initial tension is obvious, nor could it be detected by enlarging the original curves and superimposing them graphically. The diastolic arterial pressure shows no significant change and the pulse pressures are identical. The ventricular pressure curves in these segments attain the same pressure level and are superimposable, with the exception that the

diastolic decline of pressure begins somewhat earlier in the second and third segments. This is not due to accidental variation in the movement of the photosensitive paper, for calculations show that the duration of systole is reduced (0.190 > 0.175 sec.), owing to an abbreviation of systolic ejection 0.135 > 0.113 sec. These minor changes are the only ones that can possibly be referred to a *direct* effect of CO_2.

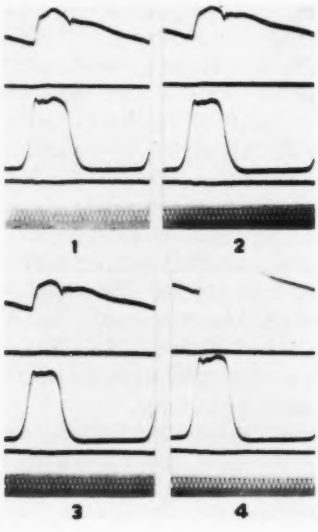


Fig. 1. Four segments of records (expt. C-455-III) during rebreathing of different percentages of CO_2. Upper curve, aortic pressure; lower, left intraventricular pressures. Time 0.02 sec. Calculations of data as follows:

SEGMENT	CO_2	ISOMETRIC CONTRACTION	EJECTION PHASE	TOTAL SYSTOLE	CYCLE	V.P.
	mm.	*seconds*	*seconds*	*seconds*	*seconds*	*mm.*
I	18+	0.055	0.135	0.190	0.405	46
II	53	0.062	0.113	0.175	0.362	45
III	60	0.062	0.113	0.175	0.360	45
IV	60				0.528	68

During these tests the natural rising tendency for venous pressure was successfully and exactly counteracted by a slight traction upon the inferior vena cava and the increasing arterial pressure was nicely compensated by loosening the aortic clamp. When during administration of high CO_2 mixtures the inferior vena cava was released and no further effort was made to control the arterial resistance, the right auricular pressure rose to

68 mm. saline and the vigor of the ventricular action increased noticeably. This is also evidenced by the record shown in segment 4. The initial tension is higher, the curve rises with a steeper gradient and reaches a higher summit. The diastolic arterial pressure is also higher and the pulse pressure is greater, indicating a greater systolic discharge. The heart rate also decreased.

A comparison of segments 3 and 4 shows clearly that the stimulating action of CO_2 is a secondary effect when the increased venous return and higher arterial resistance are permitted to come into play.

Figures 2 and 3 show two series of similar records obtained from a dog while the CO_2 content of the mixture rebreathed was first gradually increased and then slowly reduced. The venous pressures were well controlled and remained at a reasonably constant level, as shown by the data in the legends. Nevertheless, the initial left ventricular pressures were not quite constant as a glance at the records shows, a possible difficulty that Ketcham, King and Hooker (1912) foreseaw. More significant, however, was the fact that arterial pressure was not well controlled. The records show that variations in the diastolic aortic pressure occur frequently. In consequence, the reaction of the ventricle varies greatly. A casual glance gives the impression that during certain periods of the experiment, CO_2 has a tendency to stimulate and at other times a tendency to depress. A careful study of such a series of curves shows clearly, however, that it does neither by a direct action on the heart. For instance, let us compare segments 4, 6 and 7 of figure 2. In these, venous pressure, initial tension, diastolic pressure and cycle length are practically the same. These segments may therefore be used to evaluate the *direct* effects of hypercapnia. Measurement shows that the arterial pulse pressures and amplitude of the ventricular pressure curves are identical. Calculations show no demonstrable changes in duration of systole or of ejection. The only noticeable change occurs in the summits of the pressure pulses; the arch becomes less sloping during the end of ejection as CO_2 increases. But even this slight effect is probably occasioned by uncontrollable changes in arterial resistance peripheral to the aortic clamp and therefore not referable to a direct action of CO_2 on the heart.

Curves obtained in the descending series of CO_2 tensions are illustrated in figure 3. Variations in the vigor of beats also occur in successive segments; but a comparison of segments 1, 2, 5 and 6 in which all secondary factors, such as cycle length, initial tension and diastolic pressure are constant, shows again that the only significant difference is in the slope of the systolic summit of the pressure pulses. Again we cannot conclude otherwise than that the CO_2 exerts no direct effect on the dynamic action of the ventricle.

The irregular responses in other segments can all be accounted for,

and chiefly by variations in the diastolic pressure. Thus, the depression in segments 2 and 5 of figure 2 accompanies the lower diastolic aortic pressure. When as in segment 3 the diastolic pressure is increased mechanically the ventricular action improves in spite of a further increase in the intra-alveolar CO_2 tension. Similarly, the increase in diastolic pressure seen in segment 3 of figure 3 and the fall observed in segments 4 and 5 make it obvious that the dynamic action of the ventricle parallels changes in arterial resistance and not changes in CO_2. Finally, a comparison of segments 4 and 5 in which arterial diastolic pressures are comparable again show no differences.

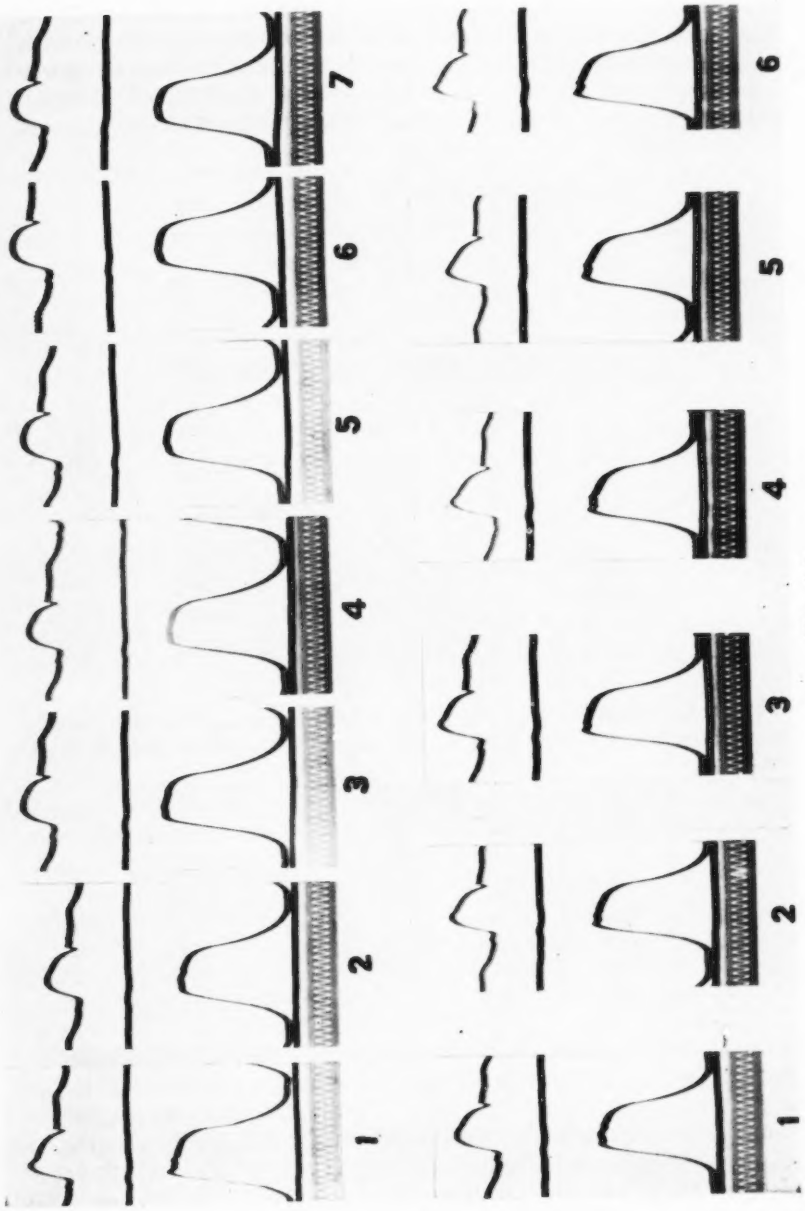
So far, the influence of changes in diastolic pressure has been especially stressed. Figure 4 includes segments of records arranged in two series in order to illustrate the importance of slight changes in initial tension. A casual glance at records in relation to CO_2 tensions noted in the legends might easily lead to the conclusion that decreasing the alveolar CO_2 produces a progressive cardiac depression in the upper set; whereas a reduction of CO_2 causes a progressive stimulation in the lower series. Again, careful analysis proves that neither represents the true effects. These records also

Fig. 2. Seven segments of records (expt. C-452-I-II) showing effects of rebreathing increasing percentages of CO_2. Upper curve, aortic pressure; lower, left ventricular pressure. Time 0.02 sec. Calculations and data as follows:

SEGMENT	CO_2	ISOMETRIC CONTRACTION	EJECTION PHASE	TOTAL SYSTOLE	CYCLE	V.P.	D.P.
	mm.	*seconds*	*seconds*	*seconds*	*seconds*	*mm.*	*mm.*
I	17.5	0.068	0.123	0.191	0.359	35	84
II	21	0.078	0.128	0.206	0.380	35	79
III	33	0.083	0.125	0.208	0.378	35	91
IV	33	0.075	0.123	0.198	0.380	35	88
V	46	0.073	0.125	0.198	0.380	35	84
VI	46	0.073	0.125	0.198	0.380	35	85
VII	53	0.075	0.123	0.198	0.380	35	85

Fig. 3. Six segments of records (expt. C-452-III) showing effects of rebreathing decreasing percentages of CO_2. Upper curve, aortic pressure; lower, left ventricular pressure. Time 0.02 sec. Calculations and data as follows:

SEGMENT	CO_2	ISOMETRIC CONTRACTION	EJECTION PHASE	TOTAL SYSTOLE	CYCLE	V.P.	D.P.
	mm.	*seconds*	*seconds*	*seconds*	*seconds*	*mm.*	*mm.*
I	57	0.06	0.118	0.178	0.348	35	77
II	53	0.06	0.115	0.175	0.350	35	77
III	44	0.063	0.120	0.183	0.348	32	82
IV	39	0.058	0.118	0.176	0.349	32	72
V	25	0.060	0.12	0.18	0.35	32	75
VI	21	0.06	0.115	0.175	0.345	32	80



Figs. 2-3

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impress the extreme caution necessary in interpreting the reactions in the intact animal whenever attempts are made to control secondary factors. The depression shown in segments 2 and 3 is partly referable to the diminished arterial pressure, but partly also to a coincident fall of the initial tension and venous pressures which naturally accompanied a reduction of

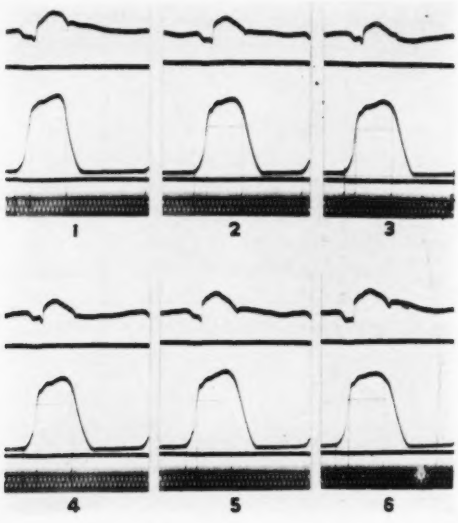

Fig. 4. Six segments of records (expt. C-454-II) during rebreathing of CO_2 (varying percentages). Upper curve, aortic pressure; lower, left ventricular pressure. Time 0.02 sec. Calculations and data as follows:

SEGMENT	CO_2	ISOMETRIC CONTRACTION	EJECTION PHASE	TOTAL SYSTOLE	CYCLE	V.P.
	mm.	*seconds*	*seconds*	*seconds*	*seconds*	*mm.*
I	60	0.068	0.162	0.230	0.590	58
II	46	0.055	0.160	0.215	0.483	42
III	39	0.047	0.167	0.214	0.489	46
IV	60	0.062	0.158	0.220	0.530	60
V	25	0.050	0.158	0.208	0.534	46
VI	21	0.054	0.159	0.213	0.517	43

CO_2. In this instance the latter changes were probably chiefly due to changes in heart rate, for it will be noticed that the cycles are shorter in segments 2 and 3 than in segment 1. The alterations are made quite obvious by comparing segments 1 and 4, in both of which CO_2 tension equalled 58 mm. In segment 4, the initial tension is reduced, the pressure curve does not rise to the previous height and the pulse pressure is less.

In segments 4 and 5 in which the beat improves, the heart cycles are of equal length. In those instances an attempt was made to neutralize the tendency of venous pressures gradually to fall by giving a slow saline infusion. Enlargement and careful superposition of the curves show a slight coincident increase in initial tension, not so obvious in reproduced records. In this instance, the small saline infusion rather than changes in CO_2 produced the stimulating effects.

Only two instances were found in the entire series of experiments in which changes in the activity of the heart could not be similarly accounted for and which may conceivably be due to a direct effect of CO_2. In one a very insignificant depression occurred (C–453–XIV); in another, an equally slight stimulation (C-455-I). The exceptional character of these reactions combined with the fact that they were opposite in direction minimizes their value in the face of consistently negative findings in the majority of experiments.

Discussion. These and many similar records lead to the conclusions 1, that increasing the CO_2 of the blood to a point until its pH is reduced approximately to 7.0 has no direct effect on the ventricular contraction; 2, that the stimulating and depressing effects noted by other observers under similar conditions are due entirely to secondary effects of CO_2 upon venous return to the right and left ventricles, upon arterial resistance and upon heart rate. The experiments of Patterson (1915), who also recorded intracardiac pressures optically, are included. This investigator interpreted his records as showing that increasing CO_2 causes a slower isometric gradient, a prolongation of systole and a slower isometric relaxation. A review of such records as are shown in figures 7 and 10 (1) of his publication confirm these statements. It may be noted, however, that the records themselves exaggerate the broadening of the pressure curves appreciably, owing to the inconstant movement of the photographic paper. Measurements of time abscissae bring out the fact that the movement was somewhat more rapid while the records labeled *b* were inscribed than when curves *a* were recorded. After making allowance, however, for this technical oversight, the pressure curves still show some broadening of contour and a slight prolongation of systole. According to my interpretation this cannot be attributed to a direct effect of CO_2 on the ventricular muscle. Close inspection of the records published shows that the heart rate slowed and that the initial tension in the ventricles rose. These facts in themselves account for the changes in the contraction curve as we have amply demonstrated in this laboratory (cf. Wiggers, 1928).

It is still necessary to harmonize my results with those of Ketcham, King and Hooker (1912), who perfused cats' hearts with Locke's solution containing 0.02 per cent $NaHCO_3$. Saturation of the solution with 3 per cent CO_2 produced no effects that, in my opinion, can be regarded as striking

when the technical difficulties of keeping all secondary factors exactly constant by such an experimental method are taken into account. Saturation of the solution with 7 per cent CO_2, on the other hand, appeared to give undoubted depressant effects. Rough calculations indicate that in such an event the pH of the perfusate must have decreased far more than in my experiments (probably to 6.4 or 6.3). Hence the possibility must be kept in mind that CO_2 may exert a direct depressant action on the heart, but that it does so only in pH ranges inconsistent with life. It would seem desirable, however, to repeat such experiments on the perfused heart, using the most perfect controls of initial intraventricular tensions and heart rate.

Finally, it remains to correlate the facts demonstrated in this and the preceding research, viz., that equal degrees of hypercapnia have no demonstrable direct effects on contraction but depress the conduction rate. Several interpretations are possible:

1. Conduction and contraction may be capable of independent alteration by changes in blood CO_2. This cannot be accepted as probable for the trend of other evidence indicates that the functions of conduction and contraction are fundamentally mediated by the same ultimate biophysical mechanisms.

2. The methods by which changes in conduction are recognized probably exceed in delicacy the best physical methods available for studying changes in contraction. Since variations in conduction-time though definite were never great, it is not improbable that the depression of contraction was so slight that it remained unrecognizable in pressure records. This must be conceded to be a possible though far from certain explanation.

3. The two methods do not necessarily compare the direct effect of CO_2 on identical portions of muscle tissue. The slight delay of conduction found in hypercapnia experiments may occur in muscle tissue of the auricle or His-Tawara system, with the A-V node as the most probable *locus*. On the other hand, the ventricular muscle concerned with contraction, in addition to conduction, may be less susceptible to changes in CO_2 concentrations within the limits attainable in intact animals. The writer favors this interpretation of the results.

In concluding, it should be reëmphasized that the results of this investigation concern the direct effect of CO_2 on ventricular contraction; they have no bearing on the effects of CO_2 on intact animals or men, i.e., when secondary influences on venous return, arterial resistance and heart rate operate in addition.

SUMMARY

1. A review of previous literature leads to the conclusion that the primary or direct effects of CO_2 on ventricular contraction have not been

crucially studied on animals with an intact circulation, the effects reported being complicated by secondary disturbances of heart rate, venous return and arterial pressures.

2. The direct effects of hypercapnia were studied on dogs whose circulation was controlled; i.e., heart rate, venous return and arterial resistance were kept constant while the animal breathed mixtures which altered the alveolar CO_2 tension between 15 and 60 mm.

3. A study of pressure curves from the left ventricle and aorta recorded by optical projection failed to show any direct effect of hypercapnia on amplitude, gradient, duration of systole or upon the pulse pressure. On the contrary, slight variations of secondary factors set in operation by increasing or decreasing CO_2 contents of the blood were capable of producing either depression or stimulation, which may easily be misinterpreted as direct effects.

BIBLIOGRAPHY

Bayliss, W. M. 1906. Ergebn. d. Physiologie, 319.

Campbell, J. M. H., C. G. Douglas, G. S. Haldane and F. G. Hobson. 1913. Journ. Physiol., xlvi, 301.

Dale, H. H. and C. L. Evans. 1922. Journ. Physiol., lvi, 125.

Eppinger, H. F. Kisch and H. Schwartz. 1927. Das Versagen des Kreislaufes, von Julius Springer, Berlin.

Gollwitzer-Meier, K. 1929. Pflüger's Arch., ccxxii, 104.

Henderson, Y. 1908. This Journal, xxi, 126.

1909. This Journal, xxiii, 345.

1910. This Journal, xxviii, 153.

Henderson, Y. and T. B. Barringer, Jr. 1913. This Journal, xxxi, 352.

Itami, S. 1912. Journ. Physiol., xlv, 338.

Jerusalem, E. and E. H. Starling. 1910. Journ. Physiol., xl, 279.

Katz, L. N. 1927. This Journal, lxxx, 470.

Ketcham, C. S., J. T. King, Jr. and D. R. Hooker. 1912. This Journal, xxxi, 64.

Kaya, R. and E. H. Starling. 1909. Journ. Physiol., xxxix, 346.

Liljestrand, G. 1919. Skand. Arch. Physiol., xxxvii, 180.

Linhard, J. 1915. Pflüger's Arch., clxi, 233.

Marshall, E. K., Jr. 1925. This Journal, lxxii, 192.

1926. Journ. Pharm. Exper. Therap., xxix, 167.

Mathison, G. C. 1910. Journ. Physiol., xli, 416.

Mines, G. R. 1913. Journ. Physiol., xlvi, 1.

Patterson, S. W. 1915. Proc. Roy. Soc., lxxxviii, 371.

Schneider, E. C. and D. Truesdell. 1922. This Journal, lxiii, 155.

Wiggers, C. J. 1927. Journ. Pharm. Exper. Therap., xxx, 218.

1928. Pressure pulses in the cardiovascular system. Longmans, Green & Co., New York and London.

Wiggers, C. J. and M. G. Banus. 1929. This Journal, xc, 218.

Wiggers, C. J. and L. N. Katz. 1928. This Journal, lxxxv, 229.